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Research paper

Pronounced anti-proliferative activity and tumor cell selectivity of 5-alkyl-2-amino-3-methylcarboxylate thiophenes

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ABSTRACT

5-(2-(4-Methoxyphenyl)ethyl)-2-amino-3-methylcarboxylate thiophene (TR560) is the prototype drug of a recently discovered novel class of tumor-selective compounds that preferentially inhibit the proliferation of specific tumor cell types (e.g. leukemia/lymphoma). Here, we further increased tumor selectivity by simplification of the molecule through replacing the 4-methoxyphenyl moiety by an alkyl chain. Several 2-amino-3-methylcarboxylate thiophene derivatives containing at C-5 an alkyl group consisting of at least 6 (hexyl) to 9 (nonyl) carbon units showed pronounced anti-proliferative activity in the mid-nanomolar range with 500- to 1000-fold tumor cell selectivity. The compounds preferentially inhibited the proliferation of T-lymphoma CEM and Molt/4, prostate PC-3, kidney Caki-1 and hepatoma Huh-7 tumor cells, but were virtually inactive against other tumor cell lines including B-lymphoma Raji and cervix carcinoma HeLa cells. The novel prototype drug **3j** (containing a 5-heptyl chain) elicited a cytotoxic, rather than cytostatic activity, already after 4 h of exposure. The unusual tumor selectivity could not be explained by a differential uptake (or efflux) of the drug by sensitive *versus* resistant tumor cells. Exposure of a fluorescent derivative of **3j** revealed pronounced uptake of the drug in the cytoplasm, no visible appearance in the nucleus, and a predominant localization in the endoplasmic reticulum. These observations may be helpful to narrow down the intracellular localization and identification of the molecular target of the 5-substituted thiophene derivatives.
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1. Introduction

Most clinically effective antitumor agents are inhibitors of DNA, RNA or protein synthesis, or interfere with the metabolism of other cell components, frequently lacking or displaying poor selective antitumor activity [1–7]. These traditional antitumor agents often poorly discriminate between tumor and normal non-tumor cells, especially those with high proliferative potential. A number of novel technologies, including antibody-drug conjugates [1], transporter-related tumor targeting of small anticancer drugs [2], prodrugs of anticancer agents [3], targeting of agents to defined (cancer) tissues [4], or specific delivery systems for anticancer drugs [5–7], have recently become subject of extensive investigations to improve anticancer selectivity.

We recently identified 5-substituted methyl 2-amino-3-carboxylate thiophenes with anti-proliferative activity against specific tumor cell lines [8,9]. The prototype compound (TR560) contains a methyl 2-amino-3-carboxylate thiophene core linked at

Abbreviations used: IC_{50}, 50% inhibitory concentration; THF, tetrahydrofuran; MeOH, methanol; EtOH, ethanol; DMF, dimethylformamide; EtOAc, ethoxyacetate; CNS, central nervous system; NBD, 7-nitrobenzofurazan or nitrobenzoxadiazole; RFP, red fluorescent protein; ER, endoplasmic reticulum; DMEM, Dulbecco's minimal essential medium; NIH, National Institutes of Health; NCI, National Cancer Institute.

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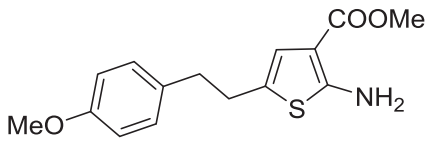


Fig. 1. Structure of 1.5-(2-(4-Methoxyphenyl)ethyl)-2-amino-3-methylcarboxylate thiophene.

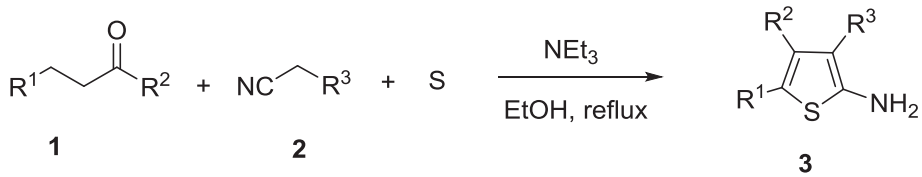
C-5 of the thiophene to a 4-methoxyphenyl through a short alkyl (i.e. ethyl) linker [8] (Fig. 1). This tumor-specific compound was originally synthesised with the aim to design and develop a novel class of antitumor agents that target tubulin [10]. The most active derivatives indeed markedly inhibited tubulin assembly and binding of radiolabeled colchicine to tubulin [10]. Most of the synthesised compounds showed pronounced anti-proliferative activity against an extensive panel of tumor cell lines, and did not discriminate between T- and B-cell lymphoma/leukemia, nor between several types of solid tumors including colon, mammary, glioma, prostate and osteosarcoma [10]. Instead, TR560 inhibited the proliferation of several human T-cell (but not B-cell lymphoma or monocyte-derived tumor cell lines), as well as several solid tumor cell lines derived from prostate (i.e. PC-3), kidney (i.e. Caki-1) and hepatoma (i.e. Huh-7) (but not colon, mammary, cervix or glioma). Moreover, the tumor-selective compound(s) showed poor, if any, anti-tubulin activity, and thus, may have acquired a more specific molecular mechanism of action. TR560 inhibited the proliferation of the sensitive tumor cell lines in the high nanomolar range while being poorly active against several other tumor cell lines (IC_{50} values in the mid-micromolar range). As a result, 60-fold tumor selectivity for T-cell lymphoma tumor cells compared to HeLa cells was obtained [8]. A slight increase in tumor selectivity could be achieved by further exploring and optimizing the substituents on the 5-phenylethyl group of the methyl 2-amino-3-carboxylate thiophene molecule [9]. In the current study, we investigated whether tumor selectivity could be further increased by replacing the 5-phenyl moiety by a methyl 2-amino-3-carboxylate thiophene thereby creating a symmetrical molecule, or by simply deleting the aromatic phenyl moiety and introducing structural variability in the aliphatic alkyl linker chain. We demonstrate that simplification of the prototype drug structure of TR560 to 5-alkyl-substituted methyl 2-amino-3-carboxylate thiophenes resulted in a substantial increase of anti-proliferative potency and markedly enhanced tumor selectivity (500- and 1400-fold for T-cell lymphoma *versus* B-cell lymphoma Raji and cervical carcinoma HeLa cells, respectively).

2. Results

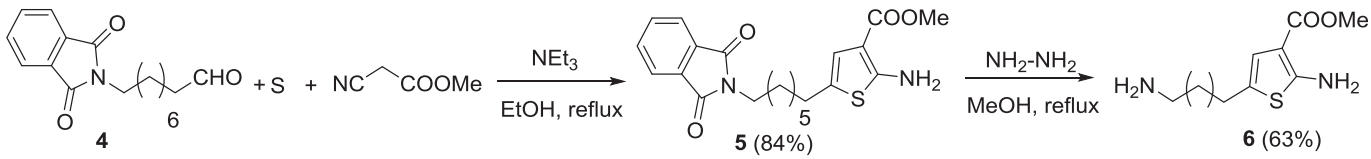
2.1. Chemistry

The Gewald three-component reaction, which results in the formation of a compound containing a multisubstituted 2-

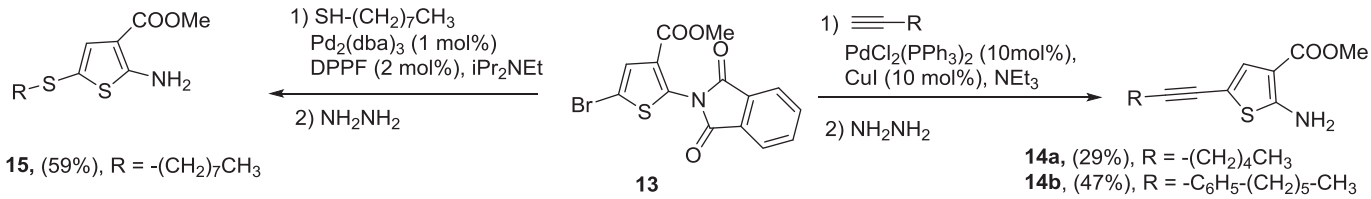
Table 1
Methyl 2-amino-5-alkylthiophene-3-carboxylate derivatives.



Entry	Compound code	R^1	R^2	R^3	Yield
1	**3a**	$-CH_3$	H	COOMe	33%
2	**3b**	$-CH_2CH_3$	H	COOMe	41%
3	**3c**	$-(CH_2)_2CH_3$	H	COOMe	53%
4	**3d**	$-(CH_2)_3CH_3$	H	COOMe	72%
5	**3e**	$-(CH_2)_4OH$	H	COOMe	56%
6	**3f**	$-(CH_2)_4CH_3$	H	COOMe	77%
7	**3g**	$-CH_2CH{=}CHCH_2CH_3$	H	COOMe	86%
8	**3h**	$-(CH_2)_5CH_3$	H	COOMe	85%
9	**3i**	$-(CH_2)_6OH$	H	COOMe	83%
10	**3j**	$-(CH_2)_6CH_3$	H	COOMe	79%
11	**3k**	$-(CH_2)_7OH$	H	COOMe	78%
12	**3l**	$-(CH_2)_3CH{=}CHCH_2CH_3$	H	COOMe	82%
13	**3m**	$-(CH_2)_6-C_6H_5$	H	COOMe	56%
14	**3n**	$-(CH_2)_7CH_3$	H	COOMe	89%
15	**3o**	$-CH(CH_3)CH_2CH_2CH{=}C(CH_3)_2$	H	COOMe	73%
16	**3p**	$-(CH_2)_7CH{=}CH_2$	H	CN	3%
17	**3q**	$-(CH_2)_7CH{=}CH_2$	H	COOMe	85%
18	**3r**	$-(CH_2)_7CH_3$	CH_3	COOMe	6%
19	**3s**	$-(CH_2)_8CH_3$	H	COOMe	92%
20	**3t**	$-(CH_2)_7C{\equiv}CH$	H	COOMe	27%
21	**3u**	$-(CH_2)_6C{\equiv}C-CH_3$	H	COOMe	85%
22	**3v**	$-(CH_2)_9CH_3$	H	COOMe	93%
23	**3w**	$-(CH_2)_9CH_2OH$	H	COOMe	76%
24	**3x**	$-(CH_2)_{10}CH_3$	H	COOMe	85%
25	**3y**	$-(CH_2)_{11}CH_3$	H	COOMe	91%
26	**3z**	$-(CH_2)_{12}CH_3$	H	COOMe	89%
27	**3aa**	$-(CH_2)_{13}CH_3$	H	COOMe	94%
28	**3ab**	$-(CH_2)_8CH{=}CH(CH_2)_3CH_3$	H	COOMe	90%
29	**3ac**	$-(CH_2)_{14}CH_3$	H	COOMe	91%
30	**3ad**	$-(CH_2)_{15}CH_3$	H	COOMe	94%

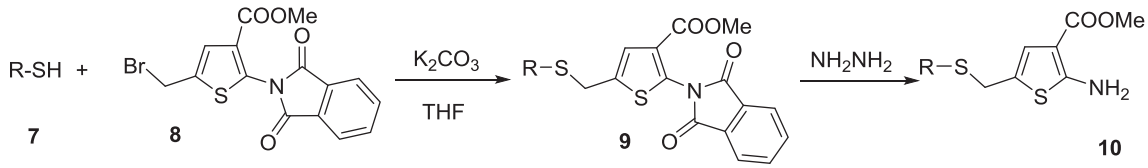


Scheme 1. Synthesis of 2-amino-5-(7-aminoheptyl)thiophene derivative **6**.



Scheme 2. Miscellaneous derivatives of 2-amino-3-carboxymethylthiophene.

Table 2
Methyl 2-amino-5-((alkylthio)methyl)thiophene-3-carboxylate derivatives.

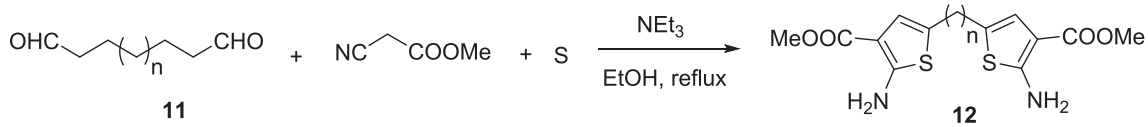


Entry	Compound code	R	Yield
1	**10a**	$-(CH_2)_3CH_3$	33%
2	**10b**	$-(CH_2)_4CH_3$	39%
3	**10c**	$-(CH_2)_2CH(CH_3)_2$	36%
4	**10d**	$-(CH_2)_5CH_3$	43%
5	**10e**	$-(CH_2)_6CH_3$	39%
6	**10f**	$-(CH_2)_7CH_3$	47%
7	**10g**	$-(CH_2)_9CH_3$	49%

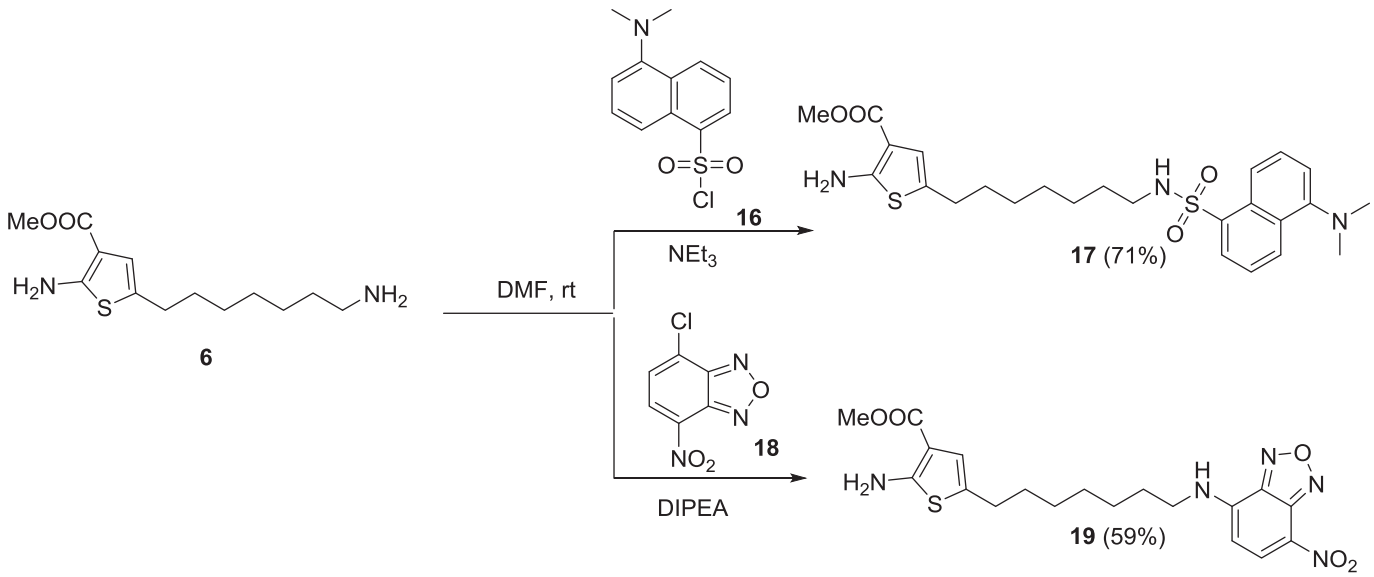
aminothiophene moiety with an electron-withdrawing entity in the 3-position has been widely applied for the synthesis and discovery of large libraries of potentially bioactive molecules [11,12]. This reaction generally involves an intermolecular Knoevenagel condensation reaction with an enolizable aldehyde and an activated nitrile followed by a base-promoted reaction with elemental sulfur followed by ring closure. The synthetic methodology applied for the preparation of the target compounds **3a-3ad** is shown in Table 1 based upon this reaction. Thus, condensation of various saturated and unsaturated aliphatic aldehydes with the methyl cyanoacetate in the presence of elemental sulfur and triethylamine yielded alkyl-2-aminothiophene-3-carboxylate derivatives **3a-3o**, **3q** and **3s-3ad** in good to excellent yields. Similarly, aminonitrile derivative **3p** was prepared by replacing methyl cyanoacetate with a malonitrile building block whereas 3-methyl aminothiophene derivative **3r** was obtained by using the corresponding ketone, decan-2-one, instead of the aldehyde derivative. However, the yields of the last two reactions were very low as compared to the other reactions.

A multi-step procedure was followed for the synthesis of 2-amino-5-(7-aminoheptyl)thiophene derivative **6**. This reaction at first involved the synthesis of phthalimido derivative **5** in good

Table 3
Bis-methyl 2-amino-5-alkylthiophene-3-carboxylate derivatives.

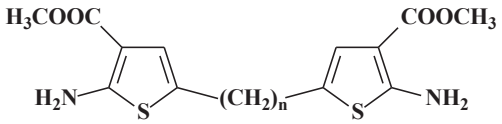


Entry	Compound code	n	Yield
1	**12a**	2	33%
2	**12b**	4	42%
3	**12c**	5	53%
4	**12d**	8	44%



Scheme 3. Synthesis of fluorescent derivatives of **6**.

Table 4
Tumor cell selectivity of 5-alkylthiophene-substituted methyl 2-amino-3-carboxylate thiophene derivatives.



Code	n	IC_{50}[a] (μM)							Ratio IC_{50}[b]	
		Lymphoma tumor cells			Carcinoma tumor cells				Raji/CEM	HeLa/PC-3
		CEM	Molt/4	Raji	PC-3	Huh-7	Caki-1	HeLa		
12a	2	5–100	65 ± 19	174 ± 6	11 ± 9	25 ± 3	41 ± 21	>100	2–35	>9
12b	4	2.4 ± 0.2	1.1 ± 0.2	83 ± 0	2.9 ± 3.6	64 ± 15	34 ± 28	52 ± 8	35	18
12c	5	0.46 ± 0.01	0.57 ± 0.51	83 ± 3	0.49 ± 0.27	25 ± 0	9.8 ± 0.5	48 ± 17	180	98
12d	8	0.76 ± 0.48	0.18 ± 0.05	58 ± 23	1.5 ± 0.4	2.4 ± 0.8	4.7 ± 2.5	50 ± 9	76	33
Prototype 1		0.90 ± 0.43	0.27 ± 0.08	57 ± 16	0.19 ± 0.04	0.95 ± 0.21	1.7 ± 1.1	39 ± 11	63	205

[a] 50%-inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%.
[b] Ratio of the IC_{50} values obtained for drug-insensitive Raji *versus* drug-sensitive CEM, or drug-insensitive HeLa *versus* drug-sensitive PC-3 tumor cells.

yield *via* Gewald reaction followed by the removal of the phthaloyl group by using hydrazine hydrate in refluxing ethanol over a period of 12 h (Scheme 1).

Next, we synthesized a series of compounds by replacing the ethylene spacer between the alkyl chain and the 2-aminothiophene of the prototype compound **3** by a thiomethylene bridge. This reaction involved the classical nucleophilic substitution reaction with various alkyl thiols and phthalimido derivative **8** [9] in the presence of K_2CO_3 as base in THF over a period of 24 h which furnished phthaloyl-protected intermediates **9a-g** in good yields. The desired compounds **10a-g** were obtained by the removal of the phthaloyl group by use of hydrazine hydrate in refluxing ethanol over a period of 12 h (Table 2).

An extension of the Gewald-multicomponent reaction where a double Gewald reaction was performed on a bifunctional building block such as enolizable bis-aliphatic aldehydes resulted in the synthesis of bis-2-aminothiophene derivatives **12a-12d** in reasonable yields (Table 3). As expected, a slightly longer reaction time is required for this reaction.

An analogue of compound **10** where the sulfur atom was directly connected at the 5-position of 2-aminothiophene moiety **15** was synthesized by utilizing an efficient $Pd(OAc)_2$/1,1'-bis(diisopropylphosphino)ferrocene-catalyzed cross-coupling reaction (Scheme 2). In order to investigate the significance of extending the ethylene spacer to an unsaturated group, the Sonogashira cross-coupling reaction has been employed on to the 5-bromo derivative of protected 2-aminothiophene **13** [10]. These reactions delivered the expected compounds **14a** and **14b** after the removal of the protecting group (Scheme 2).

Finally, fluorescent derivatives of **6** such as its dansyl derivative **17** and 7-nitrobenzofurazan derivative **19** were also synthesized by using the classical base-mediated reactions as shown in Scheme 3.

2.2. Biology

2.2.1. Structure-activity relationship of the methyl 2-amino-3-carboxylate thiophene derivatives

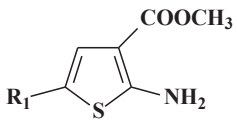
The prototype compound TR560 containing a methyl 2-amino-3-carboxylate thiophene core linked at its C-5 position to a 4-methoxy-substituted phenyl ring through an ethylene linker showed preferential anti-proliferative activity against T-cell leukemia/lymphoma compared to B-cell lymphoma cells [8]. The compound also inhibited the proliferation of several solid tumor cell lines, such as prostate PC-3, hepatoma Huh-7 and kidney Caki-1

without affecting cervix carcinoma HeLa cells [8,9]. Earlier studies revealed the crucial importance of an intact methyl 2-amino-3-carboxylate thiophene entity in the drug molecule to preserve anti-proliferative activity [8,9]. We now sought to further explore the structure-activity relationship of 5-phenylalkyl-methyl 2-amino-3-carboxylate thiophenes in which the substituted phenyl group was replaced by a methyl 2-amino-3-carboxylate thiophene entity, or methyl 2-amino-3-carboxylate thiophenes containing a simple (un)substituted aliphatic alkyl/alkenyl/alkynyl/thioalkyl chain at the C-5 position.

Given the importance of the methyl 2-amino-3-carboxylate thiophene core to preserve the anti-proliferative activity [8,9], symmetrical molecules were designed containing two methyl 2-amino-3-carboxylate thiophenes linked by an alkyl group with variable length (Table 4). When replacing the 4-methoxyphenyl group by a methyl 2-amino-3-carboxylate thiophene keeping the original ethylene linker moiety (**12a**), a substantial decrease in anti-proliferative activity was observed against the tumor cell lines tested. However, expanding the ethyl to a butyl linker (**12b**) markedly increased the anti-proliferative activity, in particular in T-lymphoma CEM and Molt/4 and in prostate cancer PC-3 cells (Table 4). Further extension of the linker to a pentyl (**12c**) or an octyl (**12d**) chain fully restored the initial tumor selectivity of the prototype compound for most tumor cell lines tested, while maintaining the anti-proliferative activity observed for TR560 (Table 4).

To reveal the importance of the second methyl 2-amino-3-carboxylate thiophene in the increased anti-proliferative activity of the symmetrical compounds, a large series of methyl 2-amino-3-carboxylate thiophene derivatives containing unsubstituted alkyl chains at the C-5 position of the thiophene ring were synthesized. Whereas the shortest methyl (**3a**) and ethyl (**3b**) substituents at C-5 did not result in relevant anti-proliferative activity, the propyl and butyl derivatives **3c** and **3d** were inhibitory in the low micromolar range (Table 5). Higher alkyl derivatives (i.e. pentyl to nonyl) (*viz.* **3f, 3h, 3j, 3n** and **3s**) were invariably inhibitory to all sensitive tumor cell lines at nanomolar concentrations. Longer alkyl chains (i.e. decyl to hexadecyl) (*viz.* **3v, 3x, 3y, 3z, 3aa, 3ab, 3ac** and **3ad**) showed a progressively lower anti-proliferative potency. Such a clear structure-anti-proliferative activity relationship was evident

Table 5
Tumor cell selectivity of 5-alkyl-substituted methyl 2-amino-3-carboxylate thiophenes.



Code	R_1	IC_{50}[a] (μM)							Ratio IC_{50}[b]	
		Lymphoma tumor cells			Carcinoma tumor cells				Raji/CEM	HeLa/PC-3
		CEM	Molt/4	Raji	PC-3	Huh-7	Caki-1	HeLa		
3a	**-CH_3**	**≥250**	**26 ± 1**	**>250**	**9.7 ± 6.3**	**94 ± 53**	**29 ± 8**	**>250**	**>1<**	**26**
3b	**-CH_2CH_3**	**175 ± 18**	**29 ± 5**	**>250**	**27 ± 1**	**84 ± 23**	**92 ± 11**	**180 ± 98**	**>1.4**	**6.7**
3c	**-$(CH_2)_2CH_3$**	**6.9 ± 2.4**	**2.1 ± 0.5**	**104 ± 3**	**3.4 ± 1.5**	**2.5 ± 0.5**	**5.4 ± 2.9**	**61 ± 1**	**16**	**18**
3d	**-$(CH_2)_3CH_3$**	**4.6 ± 1.4**	**0.90 ± 0.26**	**113 ± 4**	**4.5 ± 0.5**	**2.0 ± 1.2**	**5.6 ± 5.0**	**77 ± 2**	**25**	**17**
3e	-$(CH_2)_4OH$	58 ± 24	30 ± 0	>250	7.0 ± 5.9	45 ± 15	61 ± 52	>100	>4	>4
3f	**-$(CH_2)_4CH_3$**	**0.98 ± 0.09**	**0.13 ± 0.03**	**106 ± 0**	**0.63 ± 0.04**	**0.68 ± 0.15**	**1.4 ± 0.7**	**83 ± 13**	**108**	**132**
3g	-$CH_2CH{=}CHCH_2CH_3$	4.4 ± 1.4	1.1 ± 0.0	113 ± 7	2.5 ± 1.1	5.8 ± 1.6	15 ± 5	94 ± 10	28	38
3h	**-$(CH_2)_5CH_3$**	**0.20 ± 0.12**	**0.033 ± 0.019**	**113 ± 4**	**0.43 ± 0.03**	**0.37 ± 0.09**	**0.57 ± 0.05**	**73 ± 27**	**565**	**170**
3i	-$(CH_2)_6OH$	0.19 ± 0.01	1.4 ± 0.2	116 ± 13	0.98 ± 0.59	22 ± 7	10 ± 4	>100	611	>102
3j	**-$(CH_2)_6CH_3$**	**0.18 ± 0.08**	**0.057 ± 0.026**	**105 ± 20**	**0.62 ± 0.09**	**0.34 ± 0.09**	**0.54 ± 0.37**	**81 ± 1**	**583**	**131**
14a	-$C{\equiv}C$-$(CH_2)_4CH_3$	0.39 ± 0.06	–	–	0.42 ± 0.29	1.0 ± 0.0	1.3 ± 0.6	≥100	–	≥238
3k	-$(CH_2)_7OH$	0.074 ± 0.001	0.18 ± 0.00	109 ± 5	0.54 ± 0.35	7.0 ± 1.9	2.6 ± 0.6	≥100	1473	≥185
6	-$(CH_2)_7NH_2$	1.7 ± 1.3	0.80 ± 0.10	69 ± 1	10 ± 3	43 ± 26	12 ± 9	85 ± 9	41	8.5
3l	-$(CH_2)_3CH{=}CHCH_2CH_3$	0.25 ± 0.04	0.15 ± 0.02	99 ± 13	0.39 ± 0.21	0.63 ± 0.55	1.4 ± 0.5	88 ± 8	396	225
3m	-$(CH_2)_6$-C_6H_5	0.47 ± 0.14	0.15 ± 0.03	112 ± 25	1.2 ± 0.0	0.95 ± 0.19	1.2 ± 0.7	65 ± 3	238	54
3n	**-$(CH_2)_7CH_3$**	**0.27 ± 0.19**	**0.096 ± 0.031**	**102 ± 5**	**0.71 ± 0.09**	**0.16 ± 0.07**	**0.64 ± 0.03**	**86 ± 15**	**378**	**121**
3o	-$CH_2(CH_3)CH_2CH_2CH{=}C(CH_3)_2$	13 ± 5	2.3 ± 1.7	116 ± 8	17 ± 12	9.8 ± 4.2	26 ± 14	97 ± 2	8.9	5.7
3p[c]	-$(CH_2)_7CH{=}CH_2$	0.19 ± 0.09	0.11 ± 0.03	5.7 ± 2.4	0.22 ± 0.00	0.19 ± 0.05	0.34 ± 0.03	21 ± 5	30	95
3q	-$(CH_2)_7CH{=}CH_2$	0.28 ± 0.10	0.62 ± 0.16	>250	1.3 ± 0.7	0.74 ± 0.17	1.8 ± 1.6	60 ± 10	>893	46
3r[d]	-$(CH_2)_7CH_3$	16 ± 0	5.7 ± 1.6	125 ± 1	25 ± 0	46 ± 10	43 ± 7	84 ± 10	7.8	3.4
3s	**-$(CH_2)_8CH_3$**	**0.18 ± 0.04**	**0.058 ± 0.019**	**95 ± 38**	**0.65 ± 0.17**	**0.71 ± 0.41**	**1.2 ± 0.7**	**161 ± 125**	**528**	**248**
3t	-$(CH_2)_7C{\equiv}CH$	0.15 ± 0.02	0.10 ± 0.02	87 ± 14	0.30 ± 0.16	0.32 ± 0.23	0.55 ± 0.06	86 ± 16	580	287
3u	-$(CH_2)_6C{\equiv}C$-CH_3	0.16 ± 0.09	0.070 ± 0.049	74 ± 14	0.22 ± 0.07	0.21 ± 0.12	0.40 ± 0.02	60 ± 39	463	273
3v	**-$(CH_2)_9CH_3$**	**0.33 ± 0.20**	**0.17 ± 0.02**	**97 ± 10**	**1.2 ± 0.0**	**1.4 ± 0.4**	**3.8 ± 2.2**	**68 ± 27**	**294**	**57**
3w	-$(CH_2)_9CH_2OH$	0.16 ± 0.04	0.079 ± 0.048	86 ± 7	0.32 ± 0.10	1.7 ± 0.2	3.7 ± 2.7	43 ± 18	537	134
3x	**-$(CH_2)_{10}CH_3$**	**0.46 ± 0.01**	**0.76 ± 0.05**	**115 ± 22**	**0.54 ± 0.30**	**1.2 ± 0.1**	**0.91 ± 0.27**	**>100**	**250**	**>185**
14b	-$C{\equiv}C$-C_6H_5-$(CH_2)_5CH_3$	0.36 ± 0.03	–	–	1.5 ± 0.4	1.7 ± 0.0	4.1 ± 2.1	39 ± 1	–	30
3y	**-$(CH_2)_{11}CH_3$**	**0.56 ± 0.09**	**0.14 ± 0.03**	**109 ± 19**	**1.4 ± 0.4**	**1.3 ± 0.0**	**2.4 ± 1.7**	**187 ± 85**	**195**	**134**
3z	**-$(CH_2)_{12}CH_3$**	**1.3 ± 0.0**	**0.78 ± 0.02**	**>250**	**3.7 ± 0.3**	**4.3 ± 0.8**	**5.5 ± 4.4**	**≥250**	**>192**	**≥68**
3aa	**-$(CH_2)_{13}CH_3$**	**1.0 ± 0.3**	**-**	**-**	**1.1 ± 0.0**	**-**	**-**	**>250**	**>250**	**>227**
3ab	-$(CH_2)_8CH{=}CH(CH_2)_3CH_3$	0.22 ± 0.02	0.082 ± 0.035	98 ± 17	0.63 ± 0.02	0.47 ± 0.12	0.92 ± 0.53	55 ± 8	445	87
3ac	**-$(CH_2)_{14}CH_3$**	**6.1 ± 3.9**	**25 ± 17**	**>250**	**10 ± 1**	**40 ± 24**	**37 ± 32**	**≥250**	**>41**	**≥25**
3ad	**-$(CH_2)_{15}CH_3$**	**6.4 ± 1.7**	**5.3 ± 0.8**	**>250**	**4.8 ± 0.2**	**20 ± 9**	**3.3 ± 3.2**	**>250**	**>39**	**>52**
Prototype	**-$(CH_2)_2(4$-$(CH_3O)C_6H_5$**	**0.90 ± 0.43**	**0.27 ± 0.08**	**57 ± 16**	**0.19 ± 0.04**	**0.95 ± 0.21**	**1.7 ± 1.1**	**39 ± 11**	**63**	**205**

[a] 50%-inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%.
[b] Ratio of the IC_{50} values obtained for drug-insensitive Raji *versus* drug-sensitive CEM, or drug-insensitive HeLa *versus* drug-sensitive PC-3 tumor cells.
[c] Compound **3q** contains a cyano (-C≡N) group instead of carboxymethyl at the C-3 position of the thiophene ring.
[d] Compound **3s** contains a methyl (-CH_3) group at the C-4 position of the thiophene ring.

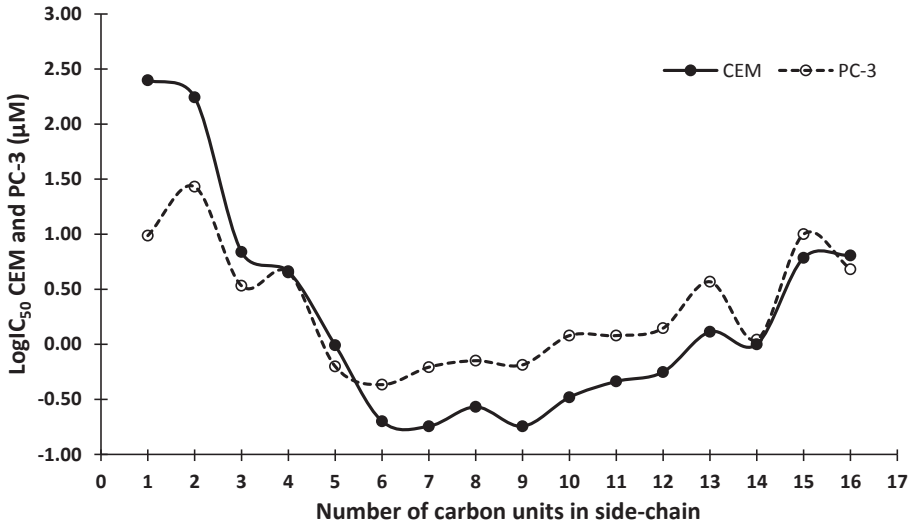


Fig. 2. Anti-proliferative activity of 5-alkyl-substituted 2-amino-3-methylcarboxylate thiophene derivatives against human T-lymphoma CEM and prostate PC-3 tumor cells.

for both T-lymphoma (i.e. CEM) and selected solid tumors (i.e. PC-3) (Fig. 2).

None of the 5-alkyl-substituted methyl 2-amino-3-carboxylate thiophenes were inhibitory against B-lymphoma Raji or cervix carcinoma HeLa cell lines, indicating a tumor selectivity profile similar to the original prototype compound. In this respect, the tumor selectivity of the most potent 5-alkyl-methyl 2-amino-3-carboxylate thiophene inhibitors (containing 5 to 9 methylene units) ranged between 108 and 580 when comparing IC_{50} values for T-lymphocyte CEM *versus* B-lymphomas Raji cells, or between 132 and 248 when comparing IC_{50} values for prostate PC-3 *versus* cervix carcinoma cells HeLa (Table 5).

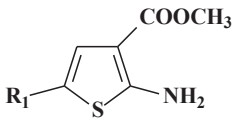
Introduction of an unsaturated alkyl chain (i.e. **3g, 3l, 3m, 3r, 3u, 3w, 3ad**) did not improve the anti-proliferative activity. Functionalization of the alkyl chain by an end-standing hydroxyl (**3e, 3i, 3k, 3w**) or amino (i.e. **6**) group resulted in variable anti-proliferative activities, i.e. decreased activity of **3e** and **3i** compared to the corresponding unsubstituted alkyl derivatives, but equal or slightly superior anti-proliferative activity of **3k** and **3w**, depending on the tumor cell line tested (Table 5).

Finally, given the improved potency and tumor selectivity of 5-alkyl derivatives and our previous findings, which demonstrated that the ethylene linker between the methyl 2-amino-3-carboxylate thiophene and the phenyl in the prototype compound could be replaced by a methylthioether entity [9], a variety of 5-alkylthiomethyl-substituted methyl 2-amino-3-carboxylate thiophene derivatives were synthesized (Table 6). The novel thioether-alkyl derivatives showed a similar tumor selectivity profile as their corresponding alkyl derivatives. However, they were endowed with comparable or inferior anti-proliferative potency compared to the 5-alkyl derivatives, resulting in lower tumor selectivity indices.

2.2.2. *Anti-proliferative activity and selectivity of compound **3j***

Based on its anti-proliferative potency and selectivity in 7 tumor cell lines (see higher), compound **3j** was chosen as the novel prototype drug for further biological studies. First, **3j** was evaluated in the NCI-60 anticancer screen of the US National Institutes of Health (NIH), containing 60 well-characterized tumor cell lines of different origins (Fig. 3). **3j** inhibited the growth of 15 cancer cell lines at

Table 6
Tumor cell selectivity of 5-alkylthiomethyl-substituted methyl 2-amino-3-carboxylate thiophene derivatives.



Code	R_1	IC_{50}[a] (µM)							Ratio IC_{50}[b]	
		Lymphoma tumor cells			Carcinoma tumor cells				Raji/CEM	HeLa/PC-3
		CEM	Molt/4	Raji	PC-3	Huh-7	Caki-1	HeLa		
10a	$-(CH_2S)(CH_2)_3CH_3$	0.52 ± 0.37	0.23 ± 0.08	112 ± 1	0.47 ± 0.26	0.40 ± 0.02	1.5 ± 0.3	81 ± 5	215	172
10b	$-(CH_2S)(CH_2)_4CH_3$	0.83 ± 0.28	0.19 ± 0.00	116 ± 6	0.94 ± 0.18	1.3 ± 0.9	3.1 ± 1.5	73 ± 11	140	78
10c	$-(CH_2S)(CH_2)_2CH(CH_3)_2$	0.91 ± 0.33	0.51 ± 0.36	132 ± 4	0.84 ± 0.15	0.86 ± 0.03	2.0 ± 0.3	71 ± 46	145	85
10d	$-(CH_2S)(CH_2)_5CH_3$	7.5 ± 3.8	1.8 ± 0.9	≥250	19 ± 2	10 ± 6	≥100	≥250	33	13
10e	$-(CH_2S)(CH_2)_6CH_3$	1.5 ± 0.0	0.29 ± 0.17	105 ± 10	3.5 ± 0.9	3.2 ± 2.2	11 ± 7	71 ± 13	70	20
10f	$-(CH_2S)(CH_2)_7CH_3$	2.9 ± 0.3	2.5 ± 0.1	120 ± 4	10 ± 5	20 ± 9	36 ± 28	>100	41	3.4
10g	$-(CH_2S)(CH_2)_9CH_3$	0.70 ± 0.04	0.22 ± 0.07	104 ± 17	2.9 ± 1.6	3.0 ± 1.8	3.1 ± 1.3	40 ± 9	149	14
15	$-(SCH_2)(CH_2)_6CH_3$	1.0 ± 0.2	0.30 ± 0.01	131 ± 5	1.5 ± 0.7	3.5 ± 0.1	1.6 ± 0.2	≥100	131	≥67
Prototype	**$-(CH_2)_2(4-(CH_3O)C_6H_5$**	**0.90 ± 0.43**	**0.27 ± 0.08**	**57 ± 16**	**0.19 ± 0.04**	**0.95 ± 0.21**	**1.7 ± 1.1**	**39 ± 11**	**63**	**205**

[a] 50%-inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%.
[b] Ratio of the IC_{50} values obtained for drug-insensitive Raji *versus* drug-sensitive CEM, or drug-insensitive HeLa *versus* drug-sensitive PC-3 tumor cells.

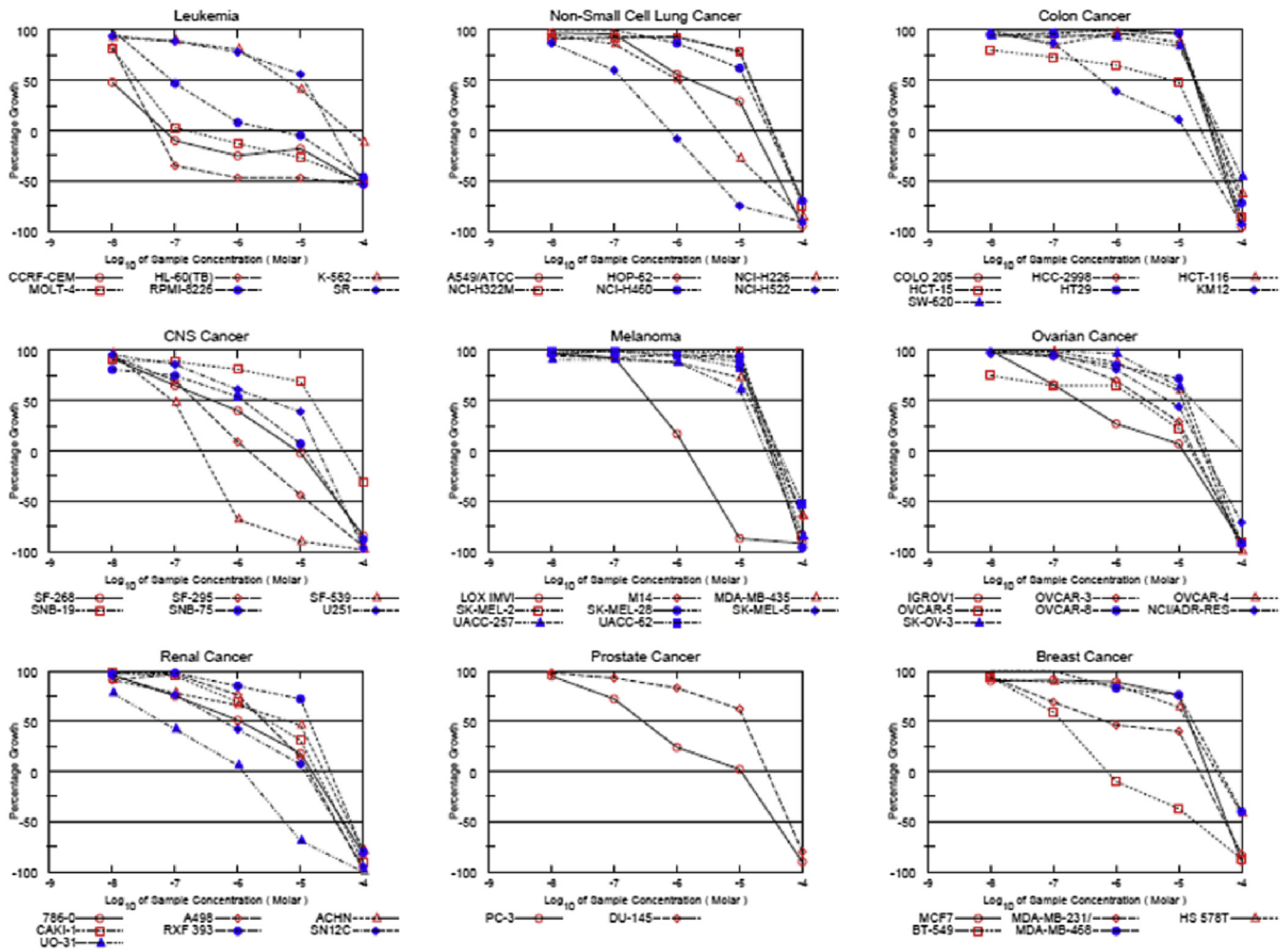


Fig. 3. Anti-proliferative activity of **3j** against a broad variety of tumor cell lines. Growth curves and drug response obtained from the NCI-60 screen (NIH).

nanomolar concentrations (IC_{50} < 1 μM). Besides leukemia/lymphoma cell lines, nanomolar anti-proliferative activity (and even cytotoxicity at 100 μM) was also detected in several renal, liver, CNS and prostate cancer cell lines. The NCI-60 screen also revealed a comparable tumor selectivity of **3j** and the initial prototype compound TR560 [8]. Finally, the selectivity profile of **3j** was not comparable to that of any known antitumor drug present in the NIH database, pointing to a likely novel molecular mechanism of action of **3j**.

We also found a high correlation (r-value between 0.85 and 0.89) between the IC_{50} values of 30 analogues of this class of compounds against T-lymphoma CEM and Molt, CEM and solid prostate PC-3, PC-3 and hepatoma Huh-7 and PC-3 and kidney Caki-1 tumor cell lines (Suppl. Fig. 1, panels A–D). These findings again argue for a similar molecular mechanism of action of the test compounds against the sensitive lymphoma and solid tumor cell types included in the study.

2.2.3. Cell growth kinetics

The IC_{50} values depicted in Tables 4–6 were calculated from the growth curves of the tumor cells as depicted in Fig. 4. Compound **3j** dose-dependently inhibited CEM (panel A) and PC-3 (panel B) tumor cell proliferation, whereas neglectable inhibitory activity was noted in HeLa cell cultures (panel C). Under these standard experimental conditions, the compound was present at a fixed concentration for the whole incubation period. Similar growth curves were found for the original prototype TR560, albeit at somewhat higher concentrations compared with **3j** (Suppl. Fig. 2, panels A–C).

Next, compound **3j** was administered to PC3 prostate cancer cell cultures for limited time periods. When exposed for 4, 2 or 1 h every day at 10 μM for up to five consecutive days, **3j** completely suppressed PC-3 proliferation upon 4 h-drug exposure, and afforded >80% and >70% suppression upon 2 h- or 1 h-drug exposure times, respectively. Also, at 2 μM **3j** (partially) suppressed tumor cell proliferation when measured at 5 days after the start of the experiment (Fig. 4, panel D). Such drug exposure schedule may somewhat mimic daily drug administration *in vivo*.

In conclusion, the tumor cell culture kinetic experiments revealed that the drug does not have to be present continuously to afford a sustainable tumor cell suppression.

2.2.4. Drug resistance selection

As already mentioned above, the identical tumor cell selectivity of the original prototype TR560 derivative and the novel prototype compound **3j** is strongly suggestive for a similar mechanism of action. To further demonstrate this, CEM and PC-3 cell cultures were exposed to escalating concentrations of TR560 or **3j** over time, starting at a ~ 2-fold IC_{50} drug value, until 10 μM-concentrations were reached (Fig. 5A). After 40 to 46 subcultivations (3–4 days for

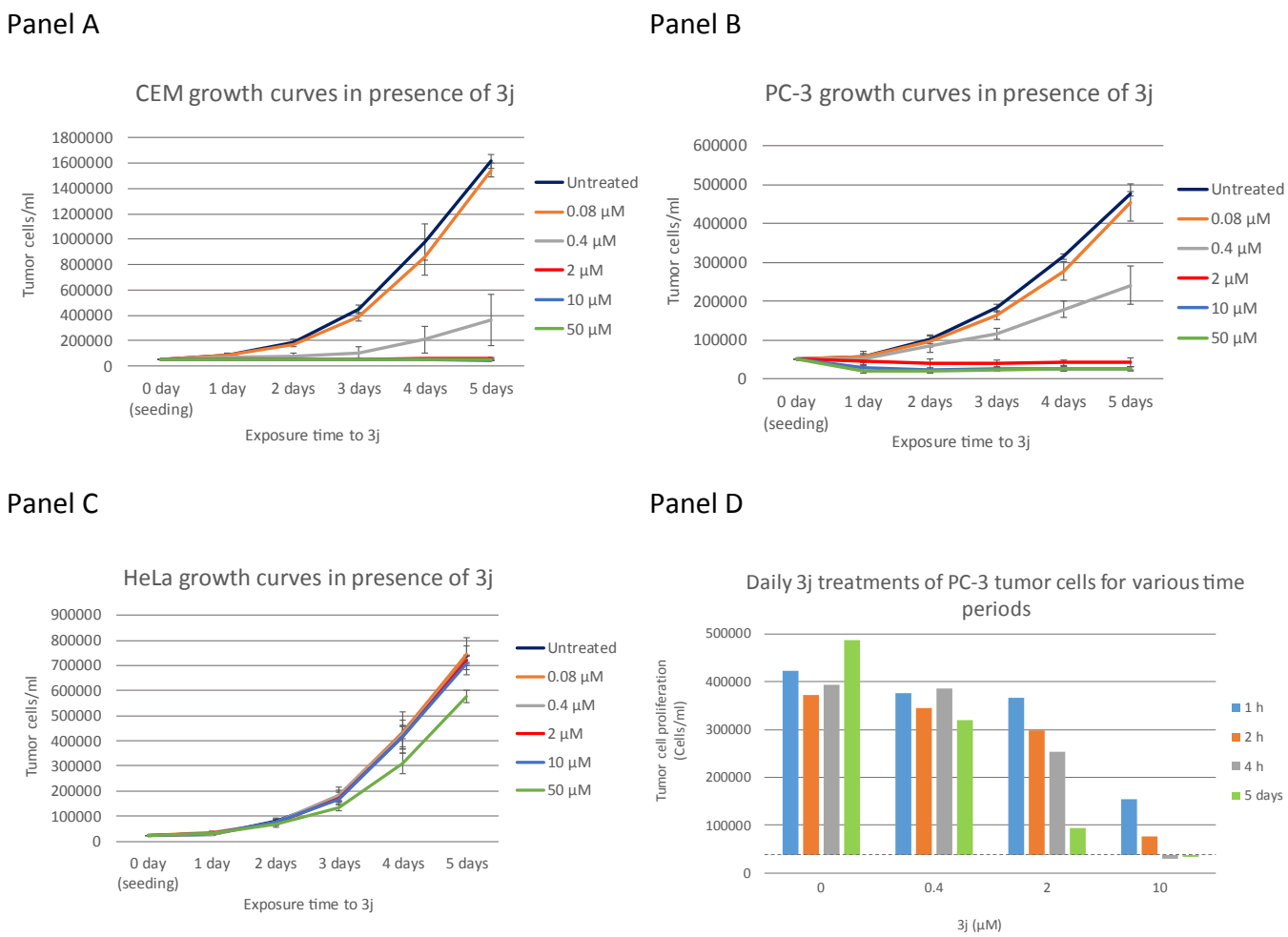


Fig. 4. Growth curves of CEM (panel A), PC-3 (panel B) and HeLa (panel C) cells in the presence of **3j**. Panel D: Effect of daily short **3j**-exposure times to PC-3 tumor cell cultures.

each passage), PC-3 and CEM cell cultures that had been exposed to escalating TR560 or **3j** concentrations became highly resistant to their selecting drug, with high cross-resistance to TR560, **3j** and **3k** (the closely related and potent 7-hydroxy derivative of **3j**) (IC_{50}: 34 to > 50 μM) (Fig. 5B and C).

2.2.5. Uptake and intracellular localization of tumor-sensitive thiophene derivatives

The molecular basis for the tumor selectivity of TR560 and **3j** is currently still unknown. To exclude differential uptake of the drugs by sensitive *versus* resistant tumor cell lines as an underlying mechanism of tumor selectivity, fluorescent derivatives of **3j**, containing a dansyl (compound **17**) or a 7-nitrobenzofurazan (NBD) moiety linked to the alkyl end of **3j** through an amine bond (compound **19**) were synthesized. Both compounds showed similar tumor selectivity properties as the parental prototype **3j**. Compound **19** was virtually equally potent in inhibiting CEM cell proliferation as **3j** (IC_{50}: 0.58 μM), **17** was somewhat less inhibitory (IC_{50}: 1 μM). However, both fluorescent compounds were markedly more inhibitory to CEM and PC-3 cells than to HeLa cells (IC_{50}: >40 μM) and thus, still kept pronounced tumor cell selectivity (Table 7). Moreover, both fluorescent compounds showed cross-reactivity with the **3j**- and **19**-resistant cells. These observations indicate that uptake and intracellular localization experiments carried out with the fluorescent drugs are reliable and relevant for their non-fluorescent prototype congeners.

In a first set of uptake experiments drug-sensitive (wild-type) CEM and PC-3 and naturally drug-resistant HeLa tumor cell cultures were exposed to **17**. In all cases, the fluorescent drug was efficiently taken-up and quickly appeared in the cytoplasm of CEM (panels A, B), PC-3 (panels C, D) and HeLa cells (panels E, F), excluding preferential uptake of the drug by the sensitive tumor cells as an underlying mechanism to explain tumor selectivity. No fluorescence was detected in the nucleus of the tumor cells (Fig. 6), even after prolonged incubation times (not shown).

In a second set of experiments, intracellular localization of **3j** was investigated using an NBD-carrying fluorescent derivative (**19**). This different fluorescent label was introduced to rule out the possibility that localization of the drug was affected by the fluorescent dansyl moiety as such. Moreover, the NBD label can be excited with visible light (but not UV light as required for dansyl excitation) avoiding cross-excitation of red fluorescent protein (see below). We focused on PC-3 cells for these studies since the cytoplasmic area of monolayer cells is much higher than that of suspension (CEM) cells. Alike the dansyl derivative **17**, the NBD fluorescent derivative **19** was abundantly taken-up into the PC-3 cytoplasm but not into the nuclear compartment.

We then exposed PC-3 cell cultures transfected with constructs expressing red fluorescent protein (RFP) conjugated to an organel-specific targeting signal, to **19**, and evaluated the localization of the red and the green signal (Fig. 7). Green fluorescence of **19** clearly did not colocalize with RFP targeted to the golgi (panels A–C),

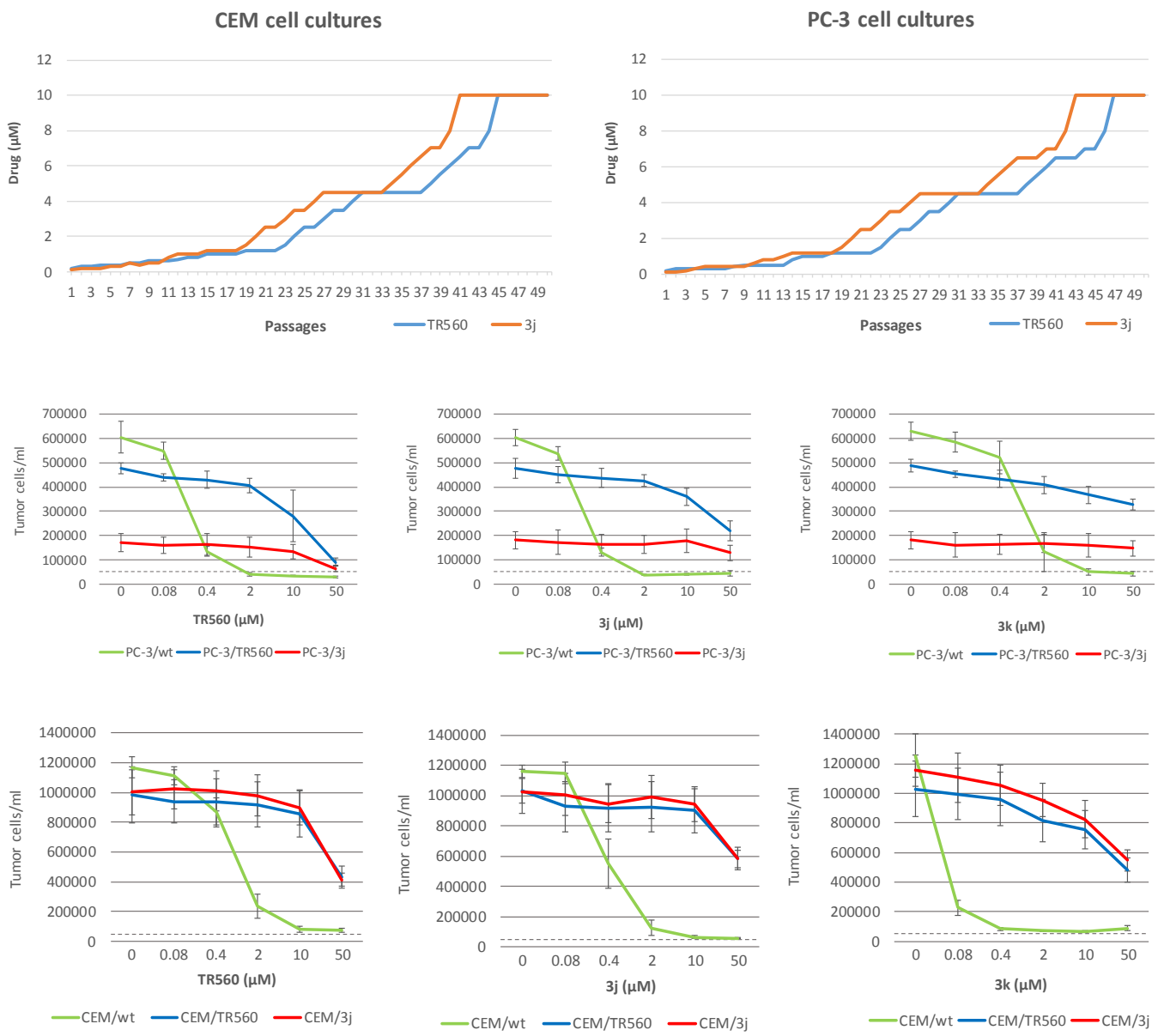


Fig. 5. Upper panels: Drug resistance selection of CEM and PC-3 cell cultures upon dose-escalating exposure to TR560 or **3j**. Middle panels: Growth curve of wild-type and drug-resistant PC-3 cell cultures in the presence of TR560, **3j** and **3k**. Lower panels: Growth curve of wild-type and drug-resistant. CEM cell cultures in the presence of TR560, **3j** and **3k**.

lysosomes (panels D–F), early endosomes (panels G–I) or late endosomes (panels J–L). While a limited overlap with RFP targeted to the mitochondria was observed (panels M–O), localization of **19** seemed to overlap to a significant extent, but not entirely, with the endoplasmic reticulum (ER) (panels P–R). Also, a rather sharp green lining around the nucleus was visible in most **19**-treated cells, pointing to its presence in, or immediately around, the nuclear membrane. Similar localization of the compound was observed in **3j**-resistant PC-3 cell cultures (Suppl. Fig. 3). These observations implicate that tumor cells that acquired resistance to the drugs by long-term exposure to escalating drug levels do not alter their drug uptake (influx) or efflux, as also observed earlier for the naturally drug-resistant HeLa cells.

Table 7
Anti-proliferative activity of fluorescent **17** and **19** against tumor cells.

	Tumor cell lines IC_{50}[a] (μM)			
	CEM	PC-3	CEM/TJ191	HeLa
17	1.0 ± 0.2	4.7 ± 1.8	7.7 ± 1.1	47 ± 7
19	0.58 ± 0.01	1.6 ± 0.0	18 ± 4	57 ± 31
3j	0.18 ± 0.08	0.62 ± 0.09	52 ± 3	81 ± 1

[a] 50% Inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%.

3. Discussion

Structure-activity relationship (SAR) studies revealed that the tumor-selective prototype drug 5-(2-(4-methoxyphenyl)ethyl)-2-amino-3-methylcarboxylate thiophene TR560 can be structurally simplified to 5-alkyl-substituted methyl 2-amino-3-carboxylate thiophenes thereby markedly increasing both the cytostatic potency and tumor selectivity (i.e. **3j**). Such 5-alkyl-thiophene derivatives have never been reported as anticancer agents, and the

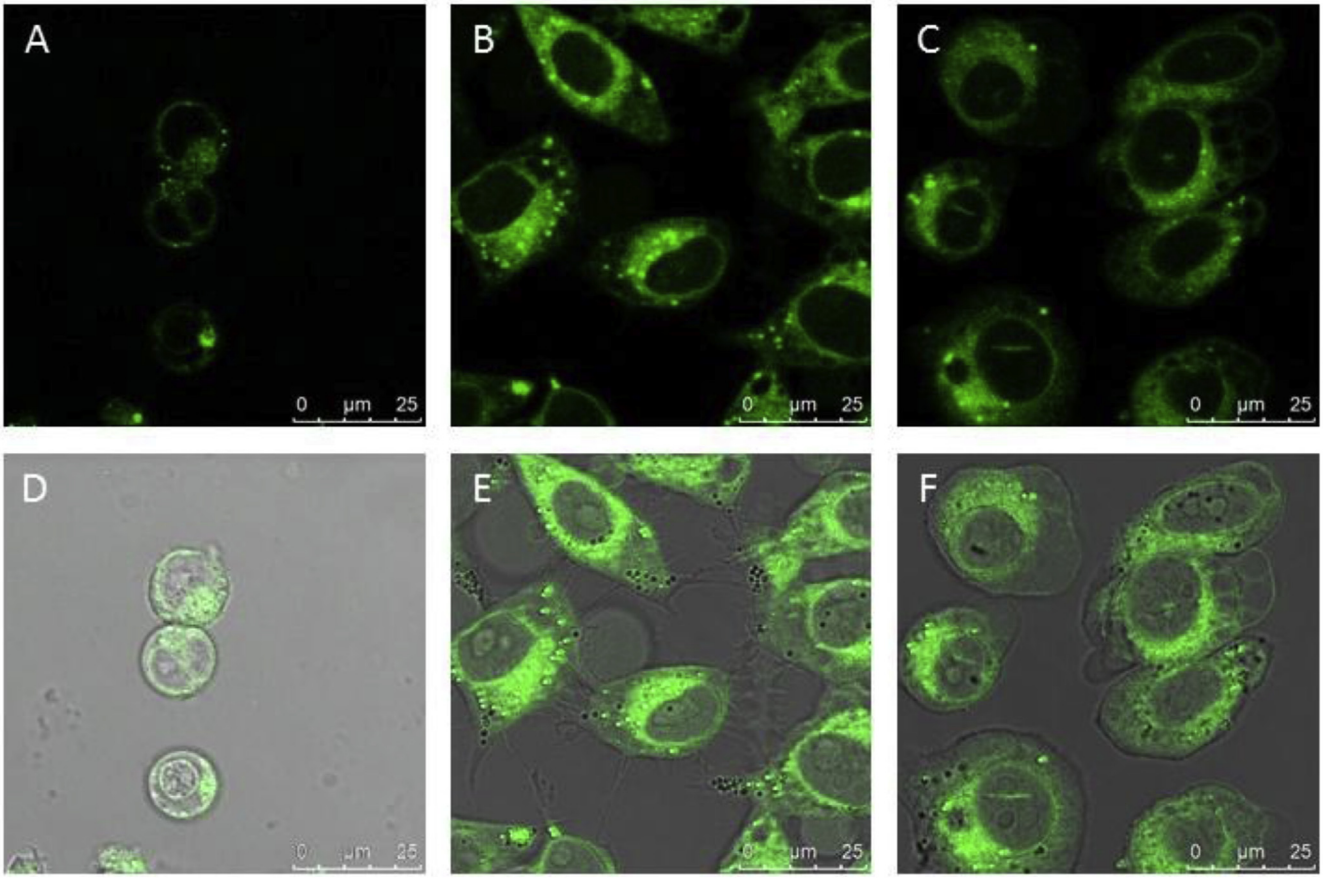


Fig. 6. Exposure of CEM, PC-3 and HeLa tumor cells to fluorescent **16**. Panels A (CEM), B (PC-3) and C (HeLa): fluorescence images. Panels D (CEM), E (PC-3) and F (HeLa): merged pictures of fluorescence- and brightfield images.

observed selective inhibition of certain well-defined tumor cell lines is quite unusual and intriguing. Moreover, unlike most cytostatic/cytotoxic drugs, **3j** did not show any toxicity in normal cells, including normal human fibroblasts (HEL; human embryonic lung fibroblasts) or blood cells (PBMC, human peripheral blood mononuclear cells) ($IC_{50} > 50$ μM, not shown). Evaluation of **3j** in the NCI-60 human tumor cell line panel revealed a unique tumor selectivity pattern, which was similar to that of the original prototype TR560 [8], but did not resemble any of the compounds included in the NCI database. This indicates that the response pattern of **3j** is unique and that the compound most likely interacts with a novel drug target. Although the 5-alkyl-2-amino-3-methylcarboxylate thiophenes contain a PAINS (Pan-Assay Interference substructure) that might affect the activity of a wide variety of proteins, thereby generating false signals across a variety of assays, and might potentially generate misleading results [15,16], it is unlikely that the data generated here are due to aspecific effects of the molecules. In fact, the compounds from which these new analogues originate target tubulin and are anti-proliferative agents that do not discriminate between different types of tumor cells [10]. Instead, the new 5-substituted 2-amino-3-methylcarboxylate thiophene derivatives do not target tubulin and showed a significant selectivity among a wide variety of tumor cell lines (Fig. 3). Such selectivity amounted up to 500- to 1500-fold for the best compounds of the series. Also, by varying the length of the 5-alkyl side chain, a close SAR could be defined for both the highly sensitive T-lymphoma (CEM) and prostate (PC-3) tumor cell lines, resulting in optimal anti-proliferative activity for an alkyl chain length between hexyl and nonyl, and markedly decreasing activities at shorter and longer alkyl side chains, pointing to a high degree of selectivity and interaction with a well-defined target. Also, although compounds with cytostatic/toxic properties were frequently shown in the past to inhibit virus replication (due to the underlying cellular toxicity, rather than a specific antiviral effect), the most active compounds did not show any measurable antiviral activity when evaluated against a broad panel of DNA and RNA viruses in at least five different cellular assay models (not shown). These observations and the above mentioned properties of the compounds let us to believe that they do not behave as PAINS. Altogether, **3j** showed cytotoxicity in almost one third of all tested cancer cell lines with over 600-fold selectivity, i.e. IC_{50} (50% inhibitory activity) of ~100 nM in sensitive tumor cell lines *versus* ~ 60 μM in insensitive tumor cell lines.

A tight correlation (r-value ranging between 0.85 and 0.89) was found between the anti-proliferative potencies of around 30 different 5-alkyl-methyl 2-amino-3-carboxylate thiophenes in T-cell lymphoma CEM *versus* Molt/4, CEM *versus* PC-3 prostate, and PC-3 *versus* kidney Caki-1 and Huh-7 hepatoma tumor cells. This suggests that the molecular target of the test compounds in leukemia/lymphoma and solid tumor cell lines is identical (Suppl. Fig. 1).

Although the molecular mechanism of action is currently unknown and subject of further studies, the fluorescently labeled thiophene derivatives were rapidly taken-up by both sensitive (CEM, PC-3) and insensitive (HeLa) tumor cells. Thus, insensitivity cannot be explained by a differential cellular drug uptake by the sensitive *versus* resistant tumor cells. Interestingly, no measurable fluorescent signal could be detected in the nucleus of both sensitive

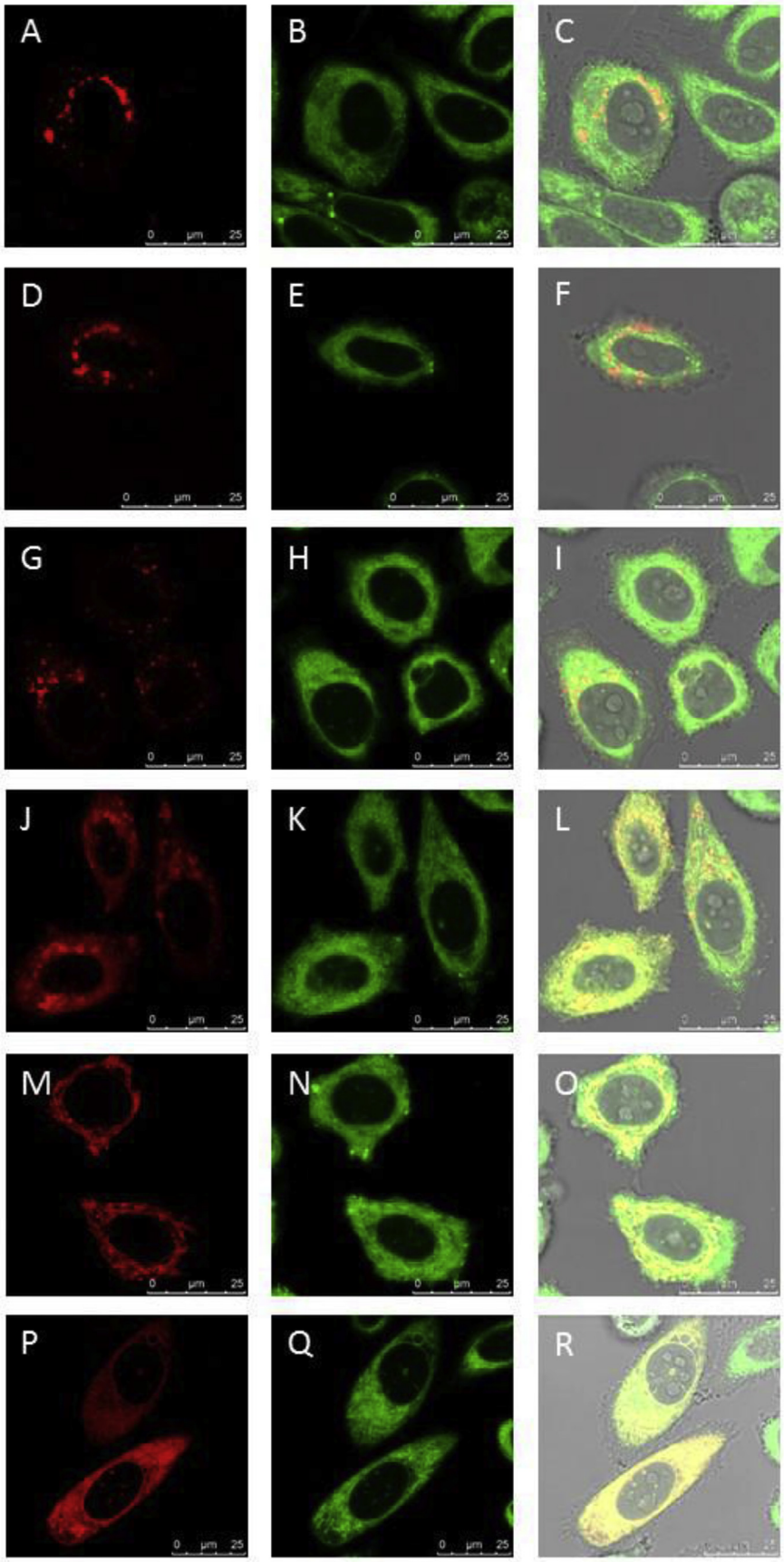


Fig. 7. Subcellular localization of organelle-targeted RFP (red) and fluorescent **3j** derivative **19** (green) in wild-type PC-3 cells. Panels A–C: A, Golgi RFP staining; B, **19** (10 μM); C, merged fluorescence overlaid brightfield image. Panels D–F: D, lysosomes RFP staining; E, **19** (10 μM); F, merged fluorescence overlaid brightfield image. Panels G–I: G, early endosomes RFP staining; H, **19** (10 μM); I, merged fluorescence overlaid brightfield image. Panels J–L: J, late endosomes RFP staining; K, **19** (10 μM); L, merged fluorescence overlaid brightfield image. Panels M–O: M, mitochondria RFP staining; N, **19** (10 μM); O, merged fluorescence overlaid brightfield image. Panels P–R: P, endoplasmatic reticulum RFP staining; Q, **19** (10 μM); R, merged fluorescence overlaid brightfield image.

and resistant tumor cell lines, while the compound was abundantly present around the nucleus and in the cytoplasm. It is therefore tempting to conclude that the eventual molecular target of the novel thiophene drugs is located outside the nucleus, excluding a wide variety of nuclear proteins and factors as potential targets. Whereas specific association/accumulation of the drug could also be excluded for a number of organelles (i.e. early and late endosomes, golgi, lysosomes, …), substantial colocalization within the ER could be observed. These observations will help to exclude/confirm intracellular targets for these thiophene derivatives.

4. Conclusions

We identified a novel class of cytostatic/toxic agents with potent selectivity against selected tumor cell types. However, investigations using the NCI-60 screen and our in-house tumor cell lines revealed that for the different organ-specific tumor cell classes, leukemia/lymphoma tumor cells display the highest chance of sensitivity to the compounds.

The molecular mechanism of tumor cell inhibition is currently unknown, but a fluorescently-labeled prototype drug revealed a fast and abundant intracellular uptake by both sensitive and resistant tumor cell lines without measurable appearance in the nuclear compartment, most likely excluding a nuclear target for these compounds.

Once the mechanism of action has been revealed and the intracellular target identified, these thiophene derivatives may qualify for a so-called tailor-made cancer drug application, to be used solely in patients who suffer from a cancer that expresses this particular molecular target. Moreover, it may be expected that, due to their inherent tumor selectivity and poor inhibitory activity against non-tumorigenic cells, these compounds will be less associated with systemic toxic side-effects than traditional chemotherapeutics. Indeed, in this respect, the new class of substituted 2-aminothiophenes differs from traditional chemotherapeutic treatment modalities that are often not selective in their antitumor activity and also affect normal cells in the tumor-bearing host.

Priority should now be given to reveal the molecular target of the compounds. This can be performed by CRISPR/Cas technology or thermal shift assay [17,18]. This will allow optimization of the lead compounds and will be helpful to better discriminate between sensitive and non-sensitive tumors.

5. Experimental section

5.1. Chemistry

5.1.1. General experimental methods

NMR spectra were acquired on commercial instruments (Bruker Avance 300 MHz, Bruker AMX 400 MHz or Bruker Avance II+ 600 MHz) and chemical shifts (δ) are reported in parts per million (ppm) referenced to tetramethylsilane (1H), or the internal (NMR) solvent signal (13C). Exact mass measurements were acquired on a Kratos MS50TC instrument (performed in the EI mode at a resolution of 10,000) or a Bruker Daltonics Apex2 FT-ICR instrument (performed in the ESI mode at a resolution of 60,000). IR spectra were recorded on a Bruker-Alpha T FTIR spectrometer with universal sampling module. Melting points (not corrected) were determined using a Reichert Thermovar apparatus. For column chromatography 70–230 mesh silica 60 (E. M. Merck) was used as the stationary phase. Chemicals received from commercial sources were used without further purification. Reaction solvents were used as received from commercial sources. The compounds purity have been estimated by HPLC. The determination of purity was conducted on a Shimadzu LC-10AT vp system with Prevail C18 column (4.6 mm × 250 mm, 5 μm). Elution was performed with a gradient of water/acetonitrile ata ratio of 50/50 for 20 min and staying at 100% acetonitrile for another 20 min. The flow rate was 1 ml/min. Peaks were detected at 254 nm. The peaks below retention time of 2.0 min and at 6.30 min were invariably observed in each of the chromotograms and is considered as an artifact but is not related to the synthesized compounds.

5.1.2. Synthesis

*5.1.2.1. General procedure for the synthesis of 2-amino-5-alkylthiophene-3-carboxylate derivatives **3** as shown in Table 1.* A solution of aliphatic carbonyl compound **1** (2 mmol), activated nitrile (2 mol), elemental sulfur (2 mmol) and triethylamine (2 mmol) in MeOH (15 ml) was heated at reflux temperature for 12 h under argon atmosphere. The MeOH was evaporated under reduced pressure and the residue was extracted with dichloromethane (3 × 50ml). The combined organic phases were washed with a saturated solution of $NaHCO_3$, dried over magnesium sulfate and the solvent was evaporated under reduced pressure. Purification by column chromatography (silica, eluent CH_2Cl_2) afforded **3** as an off-white solid or a semi-solid.

*5.1.2.2. Methyl 2-amino-5-ethylthiophene-3-carboxylate **(3b)** [13].* Off-white solid (41%, 154 mg); m. p. 46–47 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.62 (s, 1H), 5.82 (s, 2H), 3.79 (s, 3H), 2.60 (dd, J = 7.5, 0.9 Hz, 2H), 1.22 (t, J = 7.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.87, 161.47, 128.65, 120.59, 106.01, 50.99, 23.11, 15.42; Peak: 96.13%; HRMS (ESI$^+$): m/z calcd for $C_8H_{11}NO_2S$ [M+H]$^+$: 186.0583, found 186.0587.

*5.1.2.3. Methyl 2-amino-5-propylthiophene-3-carboxylate **(3c)**.* Off-white solid (53%, 212 mg); m. p. 40–41 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.61 (s, 1H), 5.71 (s, 2H), 3.79 (s, 3H), 2.55 (t, J = 7.4 Hz, 2H), 1.68–1.51 (m, 2H), 0.94 (t, J = 7.3 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.87, 161.52, 126.93, 121.52, 106.09, 51.01, 31.86, 24.37, 13.64; HRMS (ESI$^+$): m/z calcd for $C_9H_{13}NO_2S$ [M+H]$^+$: 200.0739, found 200.0733.

*5.1.2.4. Methyl 2-amino-5-butylthiophene-3-carboxylate **(3d)** [14].* Off-white solid (72%, 308 mg); m. p. 47–48 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.61 (s, 1H), 5.80 (s, 2H), 3.79 (s, 3H), 2.57 (t, J = 7.1 Hz, 2H), 1.65–1.47 (m, 2H), 1.47–1.27 (m, 2H), 0.91 (t, J = 7.3 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.88, 161.49, 127.17, 121.38, 106.08, 51.00, 33.25, 29.48, 22.13, 13.92; Peak: 99.89%; HRMS (ESI$^+$): m/z calcd for $C_{10}H_{15}NO_2S$ [M+H]$^+$: 214.0896, found 214.0899.

*5.1.2.5. Methyl 2-amino-5-(4-hydroxybutyl)thiophene-3-carboxylate **(3e)**.* Off-white solid (56%, 256 mg); m. p. 50–51 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.62 (s, 1H), 5.70 (s, 1H), 3.78 (s, 2H), 3.65 (t, J = 5.9 Hz, 2H), 2.61 (t, J = 6.6 Hz, 1H), 1.75–1.50 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.84, 161.64, 126.45, 121.69, 105.97, 62.65, 51.01, 32.00, 29.52, 27.27; Peak: 99.67%; HRMS (ESI$^+$): m/z calcd for $C_{10}H_{15}NO_3S$ [M+H]$^+$: 230.0845, found 230.0841.

*5.1.2.6. Methyl 2-amino-5-pentylthiophene-3-carboxylate **(3f)** [14].* Off-white solid (77%, 350 mg); m. p. 49–50 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.61 (s, 1H), 5.80 (s, 2H), 3.79 (s, 3H), 2.56 (t, J = 7.1 Hz, 2H), 1.70–1.48 (m, 2H), 1.43–1.22 (m, 4H), 0.89 (t, J = 6.7 Hz, 3H); Purity: 96.90%; HRMS (ESI$^+$): m/z calcd for $C_{11}H_{17}NO_2S$ [M+H]$^+$: 228.1052, found 228.1053.

*5.1.2.7. Methyl (Z)-2-amino-5-(pent-2-en-1-yl)thiophene-3-carboxylate **(3g)**.* Off-white solid (86%, 388 mg); m. p. 47–48 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.62 (s, 1H), 5.88 (s, 2H), 5.58–5.39 (m, 2H), 3.77 (s, 3H), 3.31 (d, J = 6.0 Hz, 2H), 2.30–1.94 (m, 2H), 0.99 (t,

J = 7.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.82, 161.93, 133.56, 126.06, 125.40, 121.49, 105.97, 50.95, 27.43, 20.56, 14.28; Purity: 95.21%; HRMS (ESI$^+$): m/z calcd for $C_{11}H_{15}NO_2S$ [M+H]$^+$: 226.0896, found 226.0891.

5.1.2.8. Methyl 2-amino-5-hexylthiophene-3-carboxylate ***(3h)***. Off-white solid (85%, 410 mg); m. p. 49–50 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.79 (s, 2H), 3.78 (s, 3H), 2.56 (t, J = 7.2 Hz, 2H), 1.70–1.47 (m, 2H), 1.47–1.16 (m, 6H), 0.88 (t, J = 5.9 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.88, 161.49, 127.23, 121.36, 106.08, 50.99, 31.68, 31.12, 29.82, 28.74, 22.69, 14.20; Purity: 96.23%; HRMS (ESI$^+$): m/z calcd for $C_{12}H_{19}NO_2S$ [M+H]$^+$: 242.1209, found 242.1201.

5.1.2.9. Methyl 2-amino-5-(6-hydroxyhexyl)thiophene-3-carboxylate ***(3i)***. Off-white solid (73%, 376 mg); m. p. 46–47 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.64 (s, 2H), 3.78 (s, 3H), 3.63 (t, J = 6.4 Hz, 2H), 2.57 (t, J = 7.3 Hz, 2H), 1.54–1.57 (s, 4H), 1.35–1.39 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.86, 161.56, 126.91, 121.44, 105.97, 63.00, 51.01, 32.71, 31.02, 29.69, 28.75, 25.56; Purity: 96.23%; HRMS (ESI$^+$): m/z calcd for $C_{12}H_{19}NO_3S$ [M+H]$^+$: 258.1158, found 258.1156.

5.1.2.10. Methyl 2-amino-5-heptylthiophene-3-carboxylate ***(3j)***. Off-white solid (79%, 404 mg); m. p. 44–45 °C; IR (ATR): 3425, 3309, 3167, 2925, 2850, 1655, 1500, 1445, 1257, 1191, 1129, 993, 776, 752; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.79 (s, 2H), 3.78 (s, 3H), 2.56 (t, J = 7.4 Hz, 2H), 1.74–1.39 (m, 2H), 1.44–1.16 (m, 8H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.87, 161.49, 127.22, 121.34, 106.05, 50.99, 31.87, 31.15, 29.81, 29.14, 29.02, 22.76, 14.21; Purity: 95.67%; HRMS (ESI$^+$): m/z calcd for $C_{13}H_{21}NO_2S$ [M+H]$^+$: 256.1365, found 256.1669.

5.1.2.11. Methyl 2-amino-5-(7-hydroxyheptyl)thiophene-3-carboxylate ***(3k)***. Off-white solid (78%, 424 mg); m. p. 80–81 °C; IR (ATR): 3447, 3412, 3286, 3183, 2925, 2849, 1659, 1603, 1502, 1444, 1391, 1356, 1253, 1186, 1095, 1068, 1037, 854, 779; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.76 (s, 2H), 3.78 (s, 3H), 3.63 (t, J = 6.4 Hz, 2H), 2.56 (t, J = 7.3 Hz, 2H), 1.56 (s, 4H), 1.34 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.84, 161.59, 126.93, 121.35, 105.87, 62.99, 50.97, 32.76, 30.97, 29.71, 29.18, 28.91, 25.70; Purity: 97.74%; HRMS (ESI$^+$): m/z calcd for $C_{13}H_{21}NO_3S$ [M+H]$^+$: 272.1314, found 272.1317. For ^{1}H NMR and ^{13}C NMR spectra, see also Suppl. Fig. 4.

5.1.2.12. Methyl (Z)-2-amino-5-(hept-4-en-1-yl)thiophene-3-carboxylate ***(3l)***. Off-white solid (82%, 416 mg); m. p. 46–47 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.61 (s, 1H), 5.85 (s, 1H), 5.88–5.00 (m, 1H), 3.78 (s, 1H), 2.57 (t, J = 7.5 Hz, 1H), 2.23–1.90 (m, 1H), 1.76–1.45 (m, 1H), 0.95 (t, J = 7.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.82, 161.60, 132.48, 128.26, 126.64, 121.51, 105.90, 50.93, 31.04, 29.22, 26.37, 20.63, 14.41; Purity: 97.13%; HRMS (ESI$^+$): m/z calcd for $C_{13}H_{19}NO_2S$ [M+H]$^+$: 254.1209, found 254.1211.

5.1.2.13. Methyl 2-amino-5-(6-phenylhexyl)thiophene-3-carboxylate ***(3m)***. Off-white solid (56%, 128 mg); m. p. 72–73 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40–7.08 (m, 5H), 6.60 (s, 1H), 5.78 (s, 2H), 3.78 (s, 3H), 2.69–2.50 (m, 4H), 1.765–1.55 (m, 4H), 1.39–1.30 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.85, 161.48, 142.86, 128.51, 128.35, 127.06, 125.71, 121.41, 106.06, 51.00, 36.02, 31.47, 31.03, 29.78, 29.09, 28.87; Purity: 99.18%; HRMS (ESI$^+$): m/z calcd for $C_{18}H_{23}NO_2S$ [M+H]$^+$: 318.1522, found 318.1529.

5.1.2.14. Methyl 2-amino-5-octylthiophene-3-carboxylate ***(3n)***. Off-white solid (89%, 480 mg); m. p. 45–46 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.56 (s, 1H), 5.74 (s, 2H), 3.75 (s, 3H), 2.51 (s, 2H), 1.51 (s, 2H), 1.25–1.20 (m, 10H), 0.83 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.88, 161.48, 127.24, 121.36, 106.09, 50.99, 31.98, 31.15, 29.82, 29.44, 29.33, 29.07, 22.79, 14.22; Purity: 100%; HRMS (ESI$^+$): m/z calcd for $C_{14}H_{23}NO_2S$ [M+H]$^+$: 270.1522, found 270.1523.

5.1.2.15. Methyl 2-amino-5-(6-methylhept-5-en-2-yl)thiophene-3-carboxylate ***(3o)***. Off-white solid (73%, 392 mg); m. p. 49–50 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.62 (s, 1H), 5.80 (s, 2H), 5.08 (t, J = 7.1 Hz, 1H), 3.79 (s, 3H), 2.94–2.81 (m, 2H), 2.82–2.72 (m, 1H), 1.96 (q, J = 7.4 Hz, 2H), 1.68 (s, 3H), 1.60–1.52 (m, 4H), 1.22 (d, J = 6.9 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.55, 165.93, 164.89, 161.27, 136.84, 135.59, 133.22, 131.94, 127.80, 124.18, 123.84, 120.22, 109.55, 105.90, 81.21, 80.79, 80.37, 54.58, 50.97, 42.34, 38.73, 38.50, 29.52, 26.26, 25.90, 25.83, 22.63, 21.47, 17.84; Purity: 96.70%; HRMS (ESI$^+$): m/z calcd for $C_{14}H_{21}NO_2S$ [M+H]$^+$: 268.1365, found 268.1359.

5.1.2.16. 2-Amino-5-(non-8-en-1-yl)thiophene-3-carbonitrile ***(3p)***. Off-white solid (3%, 15 mg); m. p. 42–43 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.36 (s, 1H), 5.88–5.73 (m, 1H), 5.03–4.90 (m, 2H), 4.62 (s, 2H), 2.58 (t, J = 7.4 Hz, 2H), 2.08–1.98 (m, 2H), 1.65–1.50 (m, 2H), 1.42–1.22 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.72, 139.24, 130.97, 121.19, 115.83, 114.36, 87.56, 33.88, 31.09, 29.78, 29.24, 29.09, 28.97, 28.92;; HRMS (ESI$^+$): m/z calcd for $C_{14}H_{20}N_2S$ [M+H]$^+$: 249.1419, found 249.1422.

5.1.2.17. Methyl 2-amino-5-(non-8-en-1-yl)thiophene-3-carboxylate ***(3q)***. Off-white solid (85%, 479 mg); m. p. 38–39 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.88–5.74 (m, 3H), 505–4.90 (m, 2H), 3.78 (s, 3H), 2.56 (t, J = 7.4 Hz, 2H), 2.04 (q, J = 6.8 Hz, 2H), 1.66–1.48 (m, 2H), 1.40–1.25 (s, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 95.87, 91.48, 69.30, 57.19, 51.42, 44.31, 36.14, −19.00, −36.09, −38.87, −40.19, −40.69, −40.86, −40.99; Purity: 97.69%; HRMS (ESI$^+$): m/z calcd for $C_{15}H_{23}NO_2S$ [M+H]$^+$: 282.1522, found 282.1527.

5.1.2.18. Methyl 2-amino-4-methyl-5-octylthiophene-3-carboxylate ***(3r)***. Off-white solid (6%, 34 mg); m. p. 40–41 °C ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.91 (s, 1H), 3.78 (s, 3H), 2.53 (t, J = 7.5 Hz, 2H), 2.16 (s, 3H), 1.52–1.45 (m, 2H), 1.35–1.23 (m, 10H), 0.89 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.68, 161.41, 141.80, 129.86, 120.50, 50.75, 31.99, 31.42, 29.51, 29.38, 29.18, 27.16, 22.78, 14.89, 14.22. HRMS (ESI$^+$): m/z calcd for $C_{15}H_{25}NO_2S$ [M+H]$^+$: 284.1678, found 284.1671.

. **(3s)**. Off-white solid (92%, 522 mg); m. p.56–57 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.80 (s, 2H), 3.78 (s, 3H), 2.56 (t, J = 7.4 Hz, 2H), 1.60–1.41 (m, 2H), 1.36–1.20 (m, 12H), 0.88 (t, J = 6.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.88, 161.48, 127.24, 121.36, 106.08, 50.99, 32.01, 31.15, 29.82, 29.63, 29.48, 29.43, 29.06, 22.80, 14.24; Purity: 96.95%; HRMS (ESI$^+$): m/z calcd for $C_{15}H_{25}NO_2S$ [M+H]$^+$: 284.1678, found 284.1678.

5.1.2.19. Methyl 2-amino-5-(non-8-yn-1-yl)thiophene-3-carboxylate ***(3t)***. Off-white solid (27%, 150 mg); m. p. 46–47 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.81 (s, 2H), 3.78 (s, 3H), 2.56 (t, J = 7.1 Hz, 2H), 2.18 (td, J = 7.0, 2.6 Hz, 2H), 1.94 (t, J = 2.6 Hz, 1H), 1.62–1.45 (m, 4H), 1.45–1.23 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.84, 161.49, 127.02, 121.40, 106.02, 84.82, 68.26, 50.99, 31.04, 29.75, 28.90, 28.85, 28.71, 28.51, 18.47; Purity: 96.11%; HRMS (ESI$^+$): m/z calcd for $C_{15}H_{21}NO_2S$ [M+H]$^+$: 280.1365, found 280.1367.

5.1.2.20. Methyl 2-amino-5-(non-7-yn-1-yl)thiophene-3-carboxylate ***(3u)***. Off-white solid (85%, 476 mg); m. p. 42–43 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.59 (s, 1H), 5.79 (s, 2H), 3.77 (s, 3H), 2.56 (t, J = 7.5 Hz, 3H), 2.10 (t, J = 6.9 Hz, 2H), 1.77 (t, J = 2.5 Hz, 3H), 1.68–1.49 (m, 2H), 1.49–1.26 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$)

δ 165.85, 161.48, 127.04, 121.43, 106.08, 79.39, 75.60, 51.01, 31.02, 29.76, 29.04, 28.67, 28.57, 18.82, 3.61; Purity: 100%; HRMS (ESI^+): *m/z* calcd for $C_{15}H_{21}NO_2S$ $[M+H]^+$: 280.1365, found 280.1368.

5.1.2.21. Methyl 2-amino-5-decylthiophene-3-carboxylate ***(3v)***. Off-white solid (93%, 550 mg); m. p. 42–43 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.78 (s, 2H), 3.79 (s, 3H), 2.56 (t, $J = 7.5$ Hz, 2H), 1.63–1.50 (m, 2H), 1.26 (s, 14H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.89, 161.46, 127.29, 121.38, 106.15, 51.02, 32.04, 31.17, 29.84, 29.73, 29.68, 29.49, 29.46, 29.08, 22.83, 14.26; Purity: 95.26%; HRMS (ESI^+): *m/z* calcd for $C_{16}H_{27}NO_2S$ $[M+H]^+$: 298.1835, found 298.1833.

5.1.2.22. Methyl 2-amino-5-(10-hydroxydecyl)thiophene-3-carboxylate ***(3w)***. Semi-solid (76%, 477 mg); ^1H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.85 (s, 2H), 3.78 (s, 3H), 3.63 (t, $J = 6.6$ Hz, 2H), 2.56 (t, $J = 7.4$ Hz, 2H), 1.63–1.50 (m, 4H), 1.28 (s, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.85, 161.55, 127.10, 121.33, 105.93, 63.11, 50.98, 32.86, 31.07, 29.77, 29.61, 29.50, 29.37, 28.95, 25.82; Purity: 99.17; HRMS (ESI^+): *m/z* calcd for $C_{16}H_{27}NO_3S$ $[M+H]^+$: 314.1784, found 314.1789.

5.1.2.23. Methyl 2-amino-5-undecylthiophene-3-carboxylate ***(3x)***. Off-white solid (85%, 530 mg); m. p. 36–37 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 6.77 (s, 1H), 5.72 (s, 2H), 3.78 (s, 3H), 2.56 (t, $J = 7.4$ Hz, 2H), 1.66–1.50 (m, 2H), 1.40–1.18 (s, 16H), 0.88 (t, $J = 6.7$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.89, 161.48, 127.26, 121.36, 106.10, 51.01, 32.05, 31.16, 29.83, 29.7 29.76, 29.68, 29.48, 29.08, 22.83, 14.26; Purity: 99.77%; HRMS (ESI^+): *m/z* calcd for $C_{17}H_{29}NO_2S$ $[M+H]^+$: 312.1991, found 312.1987.

5.1.2.24. Methyl 2-amino-5-dodecylthiophene-3-carboxylate ***(3y)***. Off-white solid (91%, 591 mg); m. p. 50–51 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.78 (s, 2H), 3.78 (s, 3H), 2.56 (t, $J = 7.1$ Hz, 2H), 1.61–1.53 (m, 2H), 1.40–1.22 (m, 18H), 0.89 (t, $J = 8.8$, 4.7 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.89, 161.47, 127.27, 121.37, 106.12, 51.01, 32.06, 31.17, 29.84, 29.80, 29.78, 29.68, 29.49, 29.08, 22.83, 14.26; Purity: 96.81%; HRMS (ESI^+): *m/z* calcd for $C_{18}H_{31}NO_2S$ $[M+H]^+$: 326.2148, found 326.2141.

5.1.2.25. Methyl 2-amino-5-tridecylthiophene-3-carboxylate ***(3z)***. Off-white solid (89%, 605 mg); m. p. 60–61 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.74 (s, 2H), 3.78 (s, 3H), 2.56 (t, $J = 7.2$ Hz, 2H), 1.70–1.45 (m, 2H), 1.38–1.20 (s, 20H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.89, 161.48, 127.27, 121.37, 106.11, 51.00, 32.06, 31.17, 29.82, 29.79, 29.68, 29.49, 29.08, 22.83, 14.26; Purity: 96.07%; HRMS (ESI^+): *m/z* calcd for $C_{19}H_{33}NO_2S$ $[M+H]^+$: 340.2304, found 340.2309.

5.1.2.26. Methyl 2-amino-5-tetradecylthiophene-3-carboxylate ***(3aa)***. Off-solid (94%, 665 mg); m. p. 45–46 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.79 (s, 2H), 3.78 (s, 3H), 2.56 (t, $J = 7.4$ Hz, 2H), 1.62–1.50 (m, 2H), 1.35–1.21 (s, 22H), 0.89 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.88, 161.48, 127.24, 121.35, 106.08, 51.00, 32.06, 31.16, 29.82, 29.68, 29.50, 29.08, 22.83, 14.26; Purity: 97.23%; HRMS (ESI^+): *m/z* calcd for $C_{20}H_{35}NO_2S$ $[M+H]^+$: 354.2461, found 354.2461.

5.1.2.27. Methyl (Z)-2-amino-5-(tetradec-9-en-1-yl)thiophene-3-carboxylate ***(3ab)***. Off-white solid (90%, 633 mg); m. p. 32–33 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.83 (s, 2H), 5.65–5.10 (m, 2H), 3.78 (s, 3H), 2.56 (t, $J = 7.4$ Hz, 2H), 2.10–1.94 (m, 4H), 1.64–1.52 (m, 2H), 1.44–1.20 (m, 14H), 0.90 (t, $J = 4.9$ Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 165.86, 161.50, 129.99, 129.89, 127.14, 121.33, 106.00, 50.98, 32.07, 31.13, 29.85, 29.80, 29.53, 29.43, 29.37, 29.04, 27.29, 27.03, 22.46, 14.13; Purity: 96.48%; HRMS (ESI^+): *m/z* calcd for $C_{20}H_{33}NO_2S$ $[M+H]^+$: 352.2304, found 270.1523.

5.1.2.28. Methyl 2-amino-5-pentadecylthiophene-3-carboxylate ***(3ac)***. Off-white solid (91%, 667 mg); m. p. 55–56 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.79 (s, 2H), 3.78 (s, 3H), 2.56 (t, $J = 7.3$ Hz, 3H), 1.60–1.48 (m, 2H), 1.37–1.19 (s, 24H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.89, 161.49, 127.25, 121.36, 106.09, 51.00, 32.06, 31.17, 29.83, 29.80, 29.68, 29.50, 29.09, 22.83, 14.26; Purity: 96.91%; HRMS (ESI^+): *m/z* calcd for $C_{21}H_{37}NO_2S$ $[M+H]^+$: 368.2617, found 368.2614.

5.1.2.29. Methyl 2-amino-5-hexadecylthiophene-3-carboxylate ***(3ad)***. Off-white solid (94%, 718 mg); m. p. 60–61 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 1H), 5.80 (s, 2H), 3.78 (s, 3H), 2.56 (t, $J = 7.3$ Hz, 2H), 1.61–1.52 (m, 2H), 1.37–1.18 (s, 26H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.88, 161.50, 127.23, 121.36, 106.08, 50.99, 32.06, 31.16, 29.83, 29.80, 29.68, 29.50, 29.08, 22.83, 14.25; HRMS (ESI^+): *m/z* calcd for $C_{22}H_{39}NO_2S$ $[M+H]^+$: 382.2774, found 352.2776.

5.1.2.30. Synthesis of methyl 2-amino-5-(7-(1,3-dioxoisoindolin-2-yl) heptyl)thiophene-3-carboxylate ***(5)***. A solution of **4** (1 g, 3.5 mmol), methyl cyanoacetate (350mg, 3.5 mol) and triethylamine (351mg, 3.5 mmol) in MeOH (40 ml) was heated at reflux temperature for 12 h under argon atmosphere. The MeOH was evaporated under reduced pressure and the residue was extracted with dichloromethane (3 × 50ml). The combined organic phases were washed with a saturated solution of $NaHCO_3$, dried over magnesium sulfate and the solvent was evaporated under reduced pressure. Purification by column chromatography (silica, eluent CH_2Cl_2) afforded **5** as a pale yellow solid. White solid (84%, 1.19 g); m. p. 60–61 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.94–7.77 (m, 2H), 7.76–7.64 (m, 2H), 6.59 (s, 1H), 5.81 (s, 2H), 3.78 (s, 3H), 3.67 (t, $J = 7.3$ Hz, 2H), 2.62–2.50 (m, 2H), 1.67 (dd, $J = 13.7$, 7.5 Hz, 2H), 1.56 (dd, $J = 14.1$, 7.0 Hz, 2H), 1.34 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.60, 165.85, 161.52, 133.97, 132.29, 127.00, 123.28, 121.44, 106.06, 50.97, 38.13, 30.97, 29.72, 28.97, 28.80, 28.66, 26.82. HRMS (ESI^+): *m/z* calcd for $C_{21}H_{24}N_2O_4S$ $[M+H]^+$: 401.1529, found 401.1533.

5.1.2.31. Synthesis of methyl 2-amino-5-(7-aminoheptyl)thiophene-3-carboxylate ***(6)***. To a stirred suspension of the compound **5** (1 g, 2.5 mmol), in 30 ml EtOH, hydrazine hydrate (1.5 equiv) was added. The reaction mixture was then refluxed for 3 h. After this, the reaction mixture was cooled to room temperature and keep aside for another 3 h. The resulting precipitate was filtered off and the filtrate was evaporated to dryness afforded the corresponding product **6** as a semi-solid. Semi-solid (63%, 1.19 g); ^1H NMR (400 MHz, $CDCl_3$) δ 7.94–7.77 (m, 2H), 7.76–7.64 (m, 2H), 6.59 (s, 1H), 5.81 (s, 2H), 3.78 (s, 3H), 3.67 (t, $J = 7.3$ Hz, 2H), 2.62–2.50 (m, 2H), 1.67 (dd, $J = 13.7$, 7.5 Hz, 2H), 1.56 (dd, $J = 14.1$, 7.0 Hz, 2H), 1.34 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.60, 165.85, 161.52, 133.97, 132.29, 127.00, 123.28, 121.44, 106.06, 50.97, 38.13, 30.97, 29.72, 28.97, 28.80, 28.66, 26.82; purity: 96.60%; HRMS (ESI^+): *m/z* calcd for $C_{13}H_{22}N_2O_2S$ $[M+H]^+$: 271.1474, found 271.1479.

5.1.2.32. General procedure for the synthesis of ***10***. To a mixture of **8** (300 mg, 0.79 mmol) and K_2CO_3 (0.79 mmol) in THF (10 ml) was added corresponding alky thiol **7** (0.79 mmol). After stirring the resulting mixture for another 24 h at 45 °C, the reaction mixture was added to water (30 ml). CH_2Cl_2 (30 ml) was added and the organic solution was washed with distilled water (3 × 30 ml) and then evaporated to dryness to afford the crude product mixture of **9**. This crude reaction mixture was then again dissolved in 10 ml of EtOH and hydrazine (1.2 mmol) was then added into it. The reaction

mixture was refluxed for 3 h. CH_2Cl_2 (30 ml) was added and the organic solution was washed with distilled water (3 × 30 ml), dried over $MgSO_4$, filtered, and then evaporated to dryness to afford the crude product mixture. Purification by column chromatography (silica, eluent CH_2Cl_2) afforded corresponding product **10** as a semi-solid.

*5.1.2.33. Methyl 2-amino-5-((butylthio)methyl)thiophene-3-carboxylate **(10a)**.* Semi-solid (33%, 68 mg); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.75 (s, 1H), 5.93 (s, 2H), 3.79 (s, 3H), 3.68 (s, 2H), 2.46 (t, J = 7.3 Hz, 2H), 1.68–1.48 (m, 2H), 1.44–1.28 (m, 2H), 0.90 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.70, 162.77, 123.90, 123.84, 105.55, 51.06, 31.25, 31.09, 30.93, 22.08, 13.79; Purity: 95.44%; HRMS (ESI$^+$): m/z calcd for $C_{11}H_{17}NO_2S_2$ [M+H]$^+$: 260.07734, found 260.07739.

*5.1.2.34. Methyl 2-amino-5-((pentylthio)methyl)thiophene-3-carboxylate **(10b)**.* Semi-solid (39%, 83 mg); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.75 (s, 1H), 5.93 (s, 2H), 3.79 (s, 3H), 3.69 (s, 2H), 2.45 (t, J = 7.4 Hz, 2H), 1.61–1.50 (m, 2H), 1.39–1.23 (m, 4H), 0.89 (t, J = 6.9 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.73, 162.77, 123.92, 123.87, 105.58, 51.08, 31.24, 31.15, 31.11, 28.86, 22.41, 14.08; Purity: 95.365; HRMS (ESI$^+$): m/z calcd for $C_{12}H_{19}NO_2S_2$ [M+H]$^+$: 274.0929, found 274.0921.

*5.1.2.35. Methyl 2-amino-5-((isopentylthio)methyl)thiophene-3-carboxylate **(10c)**.* Semi-solid (36%, 78 mg); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.76 (s, 1H), 5.91 (s, 2H), 3.79 (s, 3H), 3.69 (s, 2H), 2.45 (t, J = 7.8 Hz, 2H), 1.71–1.58 (m, 1H), 1.48–1.39 (m, 2H), 0.88 (t, J = 6.6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.74, 162.75, 123.96, 123.84, 105.61, 51.10, 38.17, 31.08, 29.27, 27.55, 22.42; Purity: 99.18%; HRMS (ESI$^+$): m/z calcd for $C_{12}H_{19}NO_2S_2$ [M+H]$^+$: 274.0929, found 274.0935.

*5.1.2.36. Methyl 2-amino-5-((hexylthio)methyl)thiophene-3-carboxylate **(10d)**.* Off-white solid (43%, 97 mg); m. p. 71–72 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.75 (s, 1H), 5.91 (s, 2H), 3.77 (s, 3H), 3.69 (s, 2H), 2.46 (t, J = 7.3 Hz, 2H), 1.74–1.48 (m, 2H), 1.41–1.20 (m, 6H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.75, 162.75, 123.97, 105.72, 51.08, 31.56, 31.34, 31.16, 29.20, 28.67, 22.68, 14.15; Purity: 95.33%; HRMS (ESI$^+$): m/z calcd for $C_{13}H_{21}NO_2S_2$ [M+H]$^+$: 288.1086, found 288.1088.

5.1.2.37. Methyl 2-amino-5-((heptylthio)methyl)thiophene-3-carboxylate (10e). Off-white solid (39%, 93 mg); m. p. 110–111 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.75 (s, 1H), 5.93 (s, 2H), 3.79 (s, 3H), 3.68 (s, 2H), 2.45 (t, J = 7.3 Hz, 2H), 1.62–1.54 (m, 2H), 1.39–1.23 (m, 8H), 0.87 (t, J = 6.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.72, 162.78, 123.92, 123.87, 105.56, 51.07, 31.83, 31.24, 31.10, 29.17, 28.99, 28.93, 22.72, 14.19; Purity: 97.12%; HRMS (ESI$^+$): m/z calcd for $C_{14}H_{23}NO_2S_2$ [M+H]$^+$: 302.1243, found 302.1249.

*5.1.2.38. Methyl 2-amino-5-((octylthio)methyl)thiophene-3-carboxylate **(10f)**.* Off-white solid (47%, 117 mg); m. p. 116–117 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.75 (s, 1H), 5.92 (s, 2H), 3.79 (s, 3H), 3.69 (s, 2H), 2.45 (t, J = 7.4 Hz, 2H), 1.59–1.52 (m, 2H), 1.31–1.21 (m, 10H), 0.88 (t, J = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.73, 162.75, 123.95, 105.69, 51.07, 31.95, 31.34, 31.16, 29.31, 29.23, 29.00, 22.79, 14.23; Purity: 96.11%; HRMS (ESI$^+$): m/z calcd for $C_{15}H_{25}NO_2S_2$ [M+H]$^+$: 316.1399, found 316.1391.

*5.1.2.39. Methyl 2-amino-5-((decylthio)methyl)thiophene-3-carboxylate **(10g)**.* Off-white solid (49%, 133 mg); m. p. 106–107 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.75 (s, 1H), 5.91 (s, 2H), 3.79 (s, 3H), 3.68 (s, 2H), 2.45 (t, J = 7.3 Hz, 2H), 1.62–1.48 (m, 2H), 1.38–1.22 (m, 14H), 0.87 (t, J = 6.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.73, 162.77, 123.92, 123.89, 105.58, 51.08, 32.02, 31.25, 31.10, 29.68, 29.65, 29.44, 29.35, 29.18, 28.99, 22.81, 14.25; Purity: 97.39%; HRMS (ESI$^+$): m/z calcd for $C_{17}H_{29}NO_2S_2$ [M+H]$^+$: 344.1712, found 344.1718.

*5.1.2.40. General procedure for the synthesis of bis-methyl 2-amino-5-alkylthiophene-3-carboxylate derivatives **12**.* as shown in Table 3. A solution of bis-aliphatic aldehyde **11** (1 mmol), activated nitrile (2 mmol), elemental sulfur (2 mmol) and triethylamine (2 mmol) in MeOH (15 ml) was heated at reflux temperature for 16 h under argon atmosphere. The MeOH was evaporated under reduced pressure and the residue was extracted with dichloromethane (3 × 30 ml). The combined organic phases were washed with a saturated solution of $NaHCO_3$, dried over magnesium sulfate and the solvent was evaporated under reduced pressure. Purification by column chromatography (silica, eluent CH_2Cl_2) afforded **12** as an off-white solid.

*5.1.2.41. Dimethyl 5,5'-(ethane-1,2-diyl)bis(2-aminothiophene-3-carboxylate) **(12a)**.* Off-white solid (33%, 107 mg); m. p. 53–51 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.58 (s, 2H), 5.75 (s, 4H), 3.71 (s, 6H), 2.78 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.84, 161.76, 124.78, 122.51, 106.16, 51.07, 31.48; Purity: 96.81%; HRMS (ESI$^+$): m/z calcd for $C_{14}H_{16}N_2O_4S_2$ [M+H]$^+$: 341.0624, found 341.0629.

*5.1.2.42. Dimethyl 5,5'-(butane-1,4-diyl)bis(2-aminothiophene-3-carboxylate) **(12b)**.* Off-white solid (42%, 143 mg); m. p. 51–52 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.60 (s, 2H), 3.78 (s, 6H), 2.58 (s_{br}, 4H), 1.61 (s_{br}, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.83, 161.57, 126.46, 121.66, 106.06, 51.02, 30.26, 29.49; Purity: 95.344%; HRMS (ESI$^+$): m/z calcd for $C_{16}H_{20}N_2O_4S_2$ [M+H]$^+$: 369.0931, found 369.0929.

*5.1.2.43. Dimethyl 5,5'-(pentane-1,5-diyl)bis(2-aminothiophene-3-carboxylate) (**12c**).* Off-white solid (53%, 195 mg); m. p. 45–46 °C; ^{1}H NMR (3 00 MHz, $CDCl_3$) δ 6.60 (s, 2H), 5.80 (s, 4H), 3.79 (s, 6H), 2.62–2.48 (m, 4H), 1.68–1.51 (m, 4H), 1.44–1.18 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.85, 161.51, 126.83, 121.55, 106.11, 51.02, 30.81, 29.66, 28.23; Purity: 99.12%; HRMS (ESI$^+$): m/z calcd for $C_{17}H_{22}N_2O_4S_2$ [M+H]$^+$: 383.1093, found 383.1095.

*5.1.2.44. Dimethyl 5,5'-(octane-1,8-diyl)bis(2-aminothiophene-3-carboxylate) **(12d)**.* Off-white solid (44%, 187 mg); m. p. 59–60 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.60 (s, 2H), 5.83 (s, 4H), 3.78 (s, 6H), 2.55 (t, J = 7.5 Hz, 4H), 1.54 (dd, J = 14.3, 7.1 Hz, 4H), 1.29 (s, 8H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.84, 161.53, 127.07, 121.37, 106.00, 50.97, 31.04, 29.75, 29.26, 28.91; Purity: 97.41%; HRMS (ESI$^+$): m/z calcd for $C_{20}H_{28}N_2O_4S_2$ [M+H]$^+$: 425.1563, found 425.1561.

*5.1.2.45. General procedure for the synthesis of 2-amino-5-(alkynyl) thiophene-3-carboxylate derivatives **14**.* $PdCl_2(PPh_3)_2$ (0.05 mmol), CuI (0.1 mmol), and **13** (366 mg, 1.0 mmol) were added to an oven-dried two-neck flask. The flask was evacuated and backfilled with argon (3 cycles) and then charged with THF (10.0 mL). The solution was stirred for 30 min at room temperature. Then the corresponding alkyne (1.5 mmol) and NEt_3 (3 mmol) were added by syringe. The reaction mixture was heated to 50 °C and stirred for 24 h. The reaction mixture was then allowed to reach room temperature. CH_2Cl_2 (20 ml) was added and the organic solution was washed with distilled water (3 × 20 ml) and then evaporated to dryness to afford the crude intermediate. Inorganic byproducts after this reaction were then removed by flash chromatography (silica, eluent CH_2Cl_2). This crude reaction mixture was then again dissolved in 10 ml of EtOH and hydrazine (1 equiv) was then added

into it. The reaction mixture was refluxed for 3 h. CH_2Cl_2 (30 ml) was added and the organic solution was washed with distilled water (3 × 30 ml), dried over $MgSO_4$, filtered, and then evaporated to dryness to afford the crude product mixture. Purification by column chromatography (silica, eluent CH_2Cl_2) afforded the corresponding product (**14a** or **14b**) as an off-white solid.

*5.1.2.46. Methyl 2-amino-5-(hept-1-yn-1-yl)thiophene-3-carboxylate **(14a)**.* Off-white solid (29%, 73 mg); m. p. 48–49 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.99 (s, 1H), 5.97 (s, 2H), 3.78 (s, 3H), 2.36 (t, *J* = 7.1 Hz, 2H), 1.56 (dt, *J* = 14.6, 7.1 Hz, 2H), 1.44–1.16 (m, 4H), 0.90 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.48, 162.45, 129.97, 106.25, 105.82, 92.93, 73.51, 51.23, 31.24, 28.48, 22.35, 19.76, 14.11; Purity: 97.79%; HRMS (ESI$^+$): *m*/*z* calcd for $C_{13}H_{17}NO_2S$ [M+H]$^+$: 252.1052, found 252.1056.

*5.1.2.47. Methyl 2-amino-5-((4-hexylphenyl)ethynyl)thiophene-3-carboxylate **(14b)**.* Off-white solid (47%, 160 mg); m. p. 45–46 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (d, *J* = 8.1 Hz, 2H), 7.16 (s, 1H), 7.13 (d, *J* = 8.1 Hz, 2H), 6.13 (s, 2H), 3.81 (s, 3H), 2.59 (t, 2H), 1.59 (dd, *J* = 14.2, 7.2 Hz, 2H), 1.28 (t, *J* = 8.3 Hz, 6H), 0.88 (t, *J* = 6.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.46, 163.22, 143.53, 131.32, 131.07, 128.60, 120.27, 106.66, 105.00, 91.78, 82.08, 51.31, 36.05, 31.83, 31.33, 29.05, 22.73, 14.22; Purity: 96.95%; HRMS (ESI$^+$): *m*/*z* calcd for $C_{20}H_{23}NO_2S$ [M+H]$^+$: 342.1522, found 342.1527.

*5.1.2.48. Synthesis of methyl 2-amino-5-(octylthio)thiophene-3-carboxylate **(15)**.* ($Pd_2(dba)_3$) (0.01 mmol), 1,1′-Ferrocenediyl-bis(-diphenylphosphine), (DPPF), (0.02 mmol) and **13** (192 mg, 0.53 mmol) were added to an oven-dried two-neck flask. The flask was evacuated and refilled with argon (3 cycles) and then charged with toluene (6.0 ml). The solution was stirred for 1 h at room temperature. Then the 1-octanethiol (78 mg, 0.53 mmol) and *i*Pr_2NEt (0.58 mmol) were added by syringe. The reaction mixture was heated to 100 °C and stirred for 3 h. The reaction mixture was then allowed to reach room temperature. CH_2Cl_2 (30 ml) was added and the organic solution was washed with distilled water (3 × 20 ml) and then evaporated to dryness to afford the crude intermediate. Inorganic byproducts were then removed by flash chromatography (silica, eluent CH_2Cl_2). This crude reaction mixture was then again dissolved in 5 ml of EtOH and hydrazine (1 equiv) was then added into it. The reaction mixture was refluxed for 3 h. CH_2Cl_2 (30 mL) was added and the organic solution was washed with distilled water (3 × 20 mL), dried over $MgSO_4$, filtered, and then evaporated to dryness to afford the crude product mixture. Purification by column chromatography (silica, eluent CH_2Cl_2) afforded corresponding product as a semi-solid. Off-white solid (59%, 94 mg); m. p. 36–37 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.05 (s, 1H), 6.04 (s, 2H), 3.80 (s, 3H), 2.65 (t, *J* = 7.4 Hz, 2H), 1.64–1.52 (m, 2H), 1.31 (d, *J* = 29.3 Hz, 10H), 0.88 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.43, 134.07, 115.31, 107.06, 51.22, 38.88, 31.94, 29.43, 29.31, 29.26, 28.52, 22.78, 14.24; Purity: 99.21%; HRMS (ESI$^+$): *m*/*z* calcd for $C_{14}H_{23}NO_2S_2$ [M+H]$^+$: 302.1248, found 302.1241.

*5.1.2.49. Synthesis of methyl 2-amino-5-(7-((5-(dimethylamino) naphthalene)-1-sulfonamido)heptyl)thiophene-3-carboxylate **(17)**.* A solution of 6 (0.150 mg, 0.15 mmol), dansyl chloride **16** (149, 0.15 mol) and triethylamine (15 mg, 0.15 mmol) in DMF (2 ml) was treated at room temperature for 8 h under argon atmosphere. The DMF was evaporated under reduced pressure and the residue was extracted with EtOAc (3 × 10ml). The combined organic phases were washed with a saturated solution of $NaHCO_3$, dried over magnesium sulfate and the solvent was evaporated under reduced pressure. Purification by column chromatography (silica, eluent CH_2Cl_2) afforded **17** as a red solid. Red solid (71%, 60 mg); m. p. 44–45 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 (d, *J* = 8.9 Hz, 1H), 8.33–8.26 (m, 2H), 7.64–7.41 (m, 2H), 7.20 (d, *J* = 6.0 Hz, 1H), 6.24 (s, 1H), 5.79 (s, 2H), 4.71 (t, *J* = 6.0 Hz, 1H), 3.81 (s, 3H), 3.04–2.99 (m, 2H), 2.93 (s, 6H), 2.57 (t, *J* = 7.4 Hz, 2H), 1.75–1.40 (m, 4H), 1.44–1.16 (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) 165.8, 161.6, 152.9, 135.6, 131.4, 130.8, 130.7, 130.6, 129.4, 127.3, 121.4, 124.2, 119.7, 116.1, 106.1, 50.2, 46.4, 39.3, 32.0, 31.2, 29.9, 29.2, 29.1, 29.0, 28.9; Purity: 98.43%; (ESI$^+$): *m*/*z* calcd for $C_{25}H_{33}N_3O_4S_2$ [M+H]$^+$: 504.1985, found 504.1981.

*5.1.2.50. Synthesis of methyl 2-amino-5-(7-((7-nitrobenzo[c][1,2,5] oxadiazol-4-yl)amino)heptyl)thiophene-3-carboxylate **(19)**.* A solution of **6** (200 mg, 0.75 mmol), 4-chloro-7-nitrobenzofurazan **18** (146 mg, 0.75 mol) and *N*,*N*-diisopropylethylamine (97 mg, 0.75 mmol) in DMF (5 ml) was treated at room temperature for 24 h under argon atmosphere. The DMF was evaporated under reduced pressure and the residue was extracted with EtOAc (3 × 50ml). The combined organic phases were washed with a saturated solution of $NaHCO_3$, dried over magnesium sulfate and the solvent was evaporated under reduced pressure. Purification by column chromatography (silica, eluent CH_2Cl_2) afforded **19** as a red solid. Red solid (59%, 192 mg); m. p. 44–45 °C; IR (ATR): 3462, 3351, 3281, 2930, 2851, 1676, 1580, 1398, 1314, 1296, 1186, 1120, 998, 775; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (d, *J* = 8.6 Hz, 1H), 6.63 (s, 1H), 6.26 (s, 1H), 6.19 (d, *J* = 8.7 Hz, 1H), 5.81 (s, 2H), 3.81 (s, 3H), 3.51 (dd, *J* = 13.0, 6.9 Hz, 2H), 2.60 (t, *J* = 7.0 Hz, 2H), 1.86–1.77 (m, 2H), 1.62 (s, 4H), 1.46 (dd, *J* = 13.8, 8.1 Hz, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.78, 161.45, 144.39, 144.03, 136.66, 126.75, 124.04, 121.61, 106.12, 98.66, 51.04, 44.09, 30.87, 29.69, 28.99, 28.70, 28.60, 26.90; Purity: 99.89%; HRMS (ESI$^+$): *m*/*z* calcd for $C_{19}H_{23}N_5O_5S$ [M+H]$^+$: 434.1495, found 434.1493.

5.2. Biology

5.2.1. Tumor cells

Following tumor cell lines were included in the anti-proliferative assays: human T-lymphoma CEM and Molt/4 cells, B-lymphoma Raji cells and solid prostate PC-3, kidney Caki-1, liver Huh-7 hepatoma and cervix carcinoma HeLa tumor cells.

The NCI-60 cancer cell line panel consisted of the following well-defined and characterized tumor cell lines: leukemia/lymphoma: CCRF-CEM, Molt-4, HL-60, RPMI-P226, K-562, SR; central nervous system (CNS): SF-268, SNB-19, SF-295, SNB-75, SF-539, U251; colon: Colo205; HCT-15, SW-620, HCC-2998, HT29, HCT-116, KM12; non-small cell lung: A549/ATCC, NCI-H322 M, H0P-62, NCI-H460, NCI-H225, NCI-H522; melanoma: LOX IMVI, SK-HEL-2, UACC-257, M14, SK-MEL-28, UACC-62, MDA-MB-435, SK-MEL5; prostate: PC-3, DU-145; renal: 786-D, CAKI-1, UO-31, A4989, RXF393, ACHN, SN12C; breast: MCF47, BT-549, MDA-MB-231, MDA-MB-468, HS 578T; ovarian: IGROVI, OVCAR-5, SK-OV-3, OVCAR-3, OVCAR-8, OVCAR-4, NCI/ADR-RES.

5.2.2. Anti-proliferation assays

Cells (5–7.5 × 10^4 cells) and a serial (5-fold) dilution of the test compounds were added to a 96-well microtiter plate. The cells were allowed to proliferate for 72 h–96 h (depending on the nature of the tumor cell line) at 37 °C in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter Counter (Coulter Electronics Ltd, Harpenden Herts, United Kingdom). The IC_{50} (50% inhibitory concentration) was defined as the concentration of compound that inhibited the proliferation of the tumor cells by 50%. The IC_{50} values represent the average (±standard deviation) of at least 2 to 4 independent experiments.

*5.2.3. Cell proliferation recovery upon **3j** drug removal*

PC-3 cells were seeded as described previously and allowed to adhere overnight at 37 °C. Next, the cells were exposed to different concentrations (0–50 μM) of **3j** for 1 h, 4 h, 24 h and 48 h after which they were rinsed twice with DMEM (37 °C) and further cultured in drug-free DMEM. Alternatively, the drug was left on the cell cultures until the cells were harvested and counted at 72 h after the start of drug treatment (control experiment). The experiments were performed in duplicate.

*5.2.4. Daily pulse-treatments of PC-3 cell cultures by **3j***

PC-3 cells were seeded into 96-well plates at 10,000 cells/well and allowed to adhere overnight at 37 °C. For drug treatment the culture supernatants were replaced by pre-warmed (37 °C) medium containing 0, 0.4, 2 or 10 μM **3j** in duplicates and left for 1–4 h followed by removal of the drug, rinsing with DMEM (37 °C) and addition of fresh DMEM (37 °C). Treatment was repeated every day for 4 subsequent days and the cells were harvested and counted on the fifth day. In a control experiment, the various drug concentrations were left on the tumor cell cultures for the entire duration of the experiment. The experiments were performed in duplicate.

*5.2.5. Drug resistance development through prolonged escalating drug administrations selection of PC-3- and CEM-resistant cells was performed by treatment of the tumor cell cultures with increasing concentrations of TR560 or **3j***

Therefore, cells were initially treated with 100 nM of either drug. Next, the cultures were subcultured (passaged) every 4 or 5 days. At every passage, fresh drug was added and the concentration gradually increased according to the morphological state and growth of the respective cell cultures, as monitored by light microscopy. At the end of the selection procedure, the tumor cells were maintained for at least five more subcultivations in the presence of the highest drug concentration used (10 μM).

5.2.6. Intracellular drug localization imaging

PC-3 cells were seeded into 8-well chamber slides at a density of 20.000–60,000 cells/well and allowed to adhere for 24–48 h at 37 °C. The cells were thereafter transduced with 50 baculovirus particles/cell using the CellLight®Reagents*BacMam 2.0* kit (Molecular Probes by Life technologies) to express RFP in various intracellular organelles for 16–24 h. The supernatants were subsequently discarded, the cells rinsed twice with phenol red-free DMEM (37 °C) (Life technologies) and 10 μM of NBD-labeled **3j** (**19**), prepared in phenol-red free DMEM, was added immediately prior to monitoring the fluorescence-derived RFP and **19** by confocal fluorescence microscopy.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2017.03.044.

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